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THE BLOOD PLASMA CHOLESTEROL AND PHOSPHOLIPID PHOSPHORUS IN RATS FOLLOWING PARTIAL HEPATECTOMY AND FOLLOWING LIGATION OF THE BILE DUCT*

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(Received for publication, May 1, 1936)

Thannhauser and Schaber (1) first suggested that the values of the plasma cholesterol esters might be of importance in the diagnosis of hepatic damage in humans. Recently Epstein (2) has demonstrated that parenchymatous disease of the liver in man is associated generally with a marked reduction of the cholesterol esters of the blood. He further found that obstruction of the common bile duct in patients was accompanied by an increase in the total blood cholesterol and usually by an absolute increase in the cholesterol esters. There has been little work in experimental animals with impaired liver function to compare with the results obtained in patients with hepatic damage. It is the purpose of this investigation to study the effect on cholesterol and phospholipid metabolism of rats (1) following partial hepatectomy and (2) following ligation of the bile duct.

Methods

Albino rats of Wistar stock, raised in this laboratory, were used as experimental animals. The animals were between 90 and 100 days of age at the time of operation. Partial hepatectomy was performed according to the technique of Higgins and Anderson (3), thus removing 65 to 75 per cent of the total liver tissue. The ligation of the bile duct was carried out according to the method of Richter and Benjamin (4). Ether anesthesia was used and no

* This investigation was supported in part by the Josiah Macy, Jr., Foundation.

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attempt was made to use aseptic precautions. Laparotomy was performed in the control animals.

The animals were fed on a stock diet¹ and were allowed food up to the time they were sacrificed. Blood was drawn from the abdominal aorta under ether anesthesia in the majority of animals. In the animals with ligated bile ducts surviving for about 2 weeks, blood was drawn by cardiac puncture or from the carotid artery, since considerable amounts of fluid were frequently present in the abdominal cavity. The blood was prevented from clotting by use of potassium oxalate.

The micromethod of Schoenheimer and Sperry (5) was used for determining the free and esterified cholesterol. The procedures for extraction, saponification, precipitation with digitonin, and development of color were rigidly followed. A Bausch and Lomb spectrophotometer was used in place of the Pulfrich photometer. Special cells (made by Bausch and Lomb) 5 cm. in length, holding about 2 cc., were used for holding the solution. The specific extinction coefficient for cholesterol was determined at a wavelength of 615 $m\mu$ and an average value of 1.400 was obtained and used in the calculation. Phosphatide phosphorus was determined according to the procedure recommended by Man and Peters (6).

Results

Partial Hepatectomy—The individual results for free and esterified cholesterol and the percentage of esterified cholesterol are presented in Fig. 1. The mean values for the free and combined cholesterol for twenty-six control animals were 22.6 and 51.7 mg. per 100 cc. of plasma, respectively; the esterified cholesterol was 70 per cent of the total cholesterol. On the 1st day after operation, the free cholesterol remained normal, with a mean value of 27.5 mg. per cent, but there was a marked decrease in the esterified cholesterol to 27.3 mg. per cent (fourteen rats). The esterified cholesterol decreased to a mean value of 50 per cent of the total cholesterol. On the 2nd day, the average concentration of the free cholesterol increased markedly to 39.1 mg. per cent. In thirteen of the seventeen rats, the concentration of the esterified cholesterol returned to within the normal range. On the 3rd day,

¹ Bal Ra, purchased from Valentine's Meat Juice Company, Richmond, Virginia.

the free and esterified cholesterol and the percentage of cholesterol esters returned to within the normal range. Except for two animals, all the values after this time remained normal.

Ligation of the Bile Duct—The individual results for the free and esterified cholesterol and the percentage of combined cholesterol are presented in Fig. 2. It can be seen that most of the

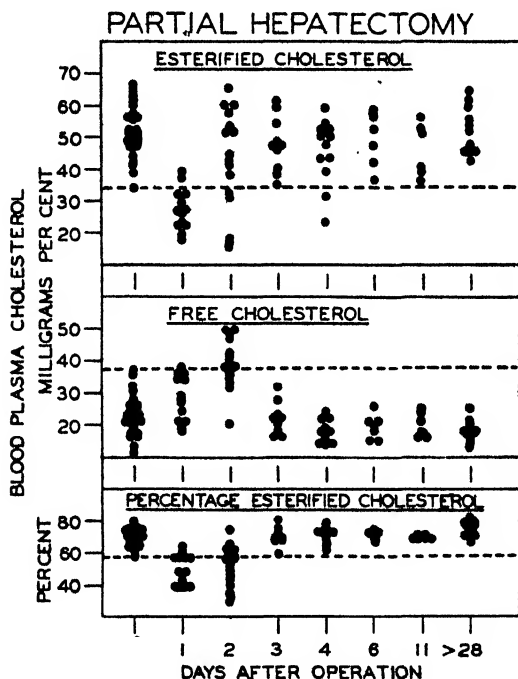


FIG. 1. Effect of partial hepatectomy on blood plasma cholesterol

esterified values vary within normal limits. Of the 111 values in experimental rats for esterified cholesterol, eleven were below the minimum normal and thirty-four were above the maximum normal: the lowest and the highest concentrations were 15 and 109 mg. per cent, respectively. The values for free cholesterol are above the normal range in the majority of instances. The percentage of esterified cholesterol was below normal in most cases

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because the ester fraction did not increase proportionately with the free cholesterol. It is interesting to note that there are such marked increases during the first few days after ligation. The highest concentrations were obtained about 2 weeks after operation.

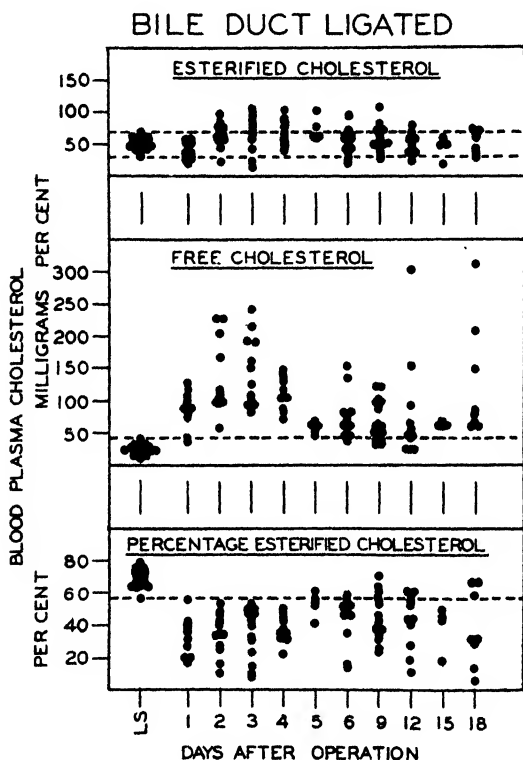


FIG. 2. Effect of ligation of bile duct on blood plasma cholesterol

In those animals in which ligation appeared to be successful, a marked jaundice was evident on the 1st day. There was a progressively increasing dilatation of the duct with time. After the 9th day the duct was markedly dilated by a greenish bile or a bloody bile mixture. There was no consistency in the analytical results in individual animals that appeared to have the same amount of

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jaundice and bile duct dilatation. A number of animals showed only a small amount of dilatation of the bile duct a week or more after ligation without any sign of cholemia. These animals must have had patent accessory ducts (4), since it was found at autopsy that the duct was transected and the cholesterol values were normal. The mortality in this group was greatest about 1 week after ligation.

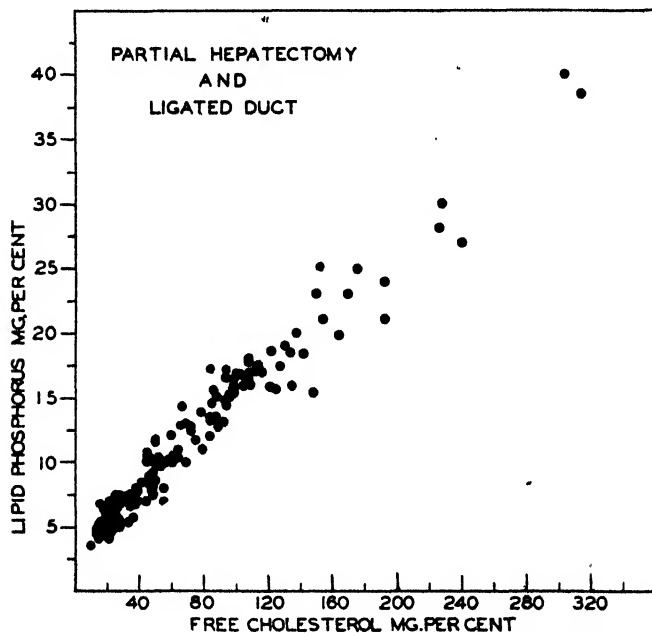


FIG. 3. Effect of partial hepatectomy and ligation of bile duct on relation of phospholipid and free cholesterol.

Relation of Phospholipid and Free Cholesterol—The individual plasma phosphatide phosphorus and free cholesterol concentrations in the partially hepatectomized and ligated duct groups are presented in Fig. 3. It is seen that there is a direct proportionality between these two constituents.

Comment

The removal of about 70 per cent of the total liver tissue in the rat caused a definite decrease in the cholesterol esters of the blood

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plasma 24 hours after operation. According to Higgins and Anderson (3) the regeneration of liver tissue at this time is not appreciable. In addition, the water content of the liver remnant is markedly reduced, which is probably due to the immediate shock of the operation. Presumably the dehydration of the liver remnant reduces its function and accounts for the decrease in cholesterol esters. With increased water content and increased mitotic activity of the liver remnant (3), the esters return to normal on the 2nd and 3rd days after operation. The evidence obtained in the partially hepatectomized rats appears to confirm the results obtained in patients with hepatic damage.

The blood plasma cholesterol values obtained in the rats with ligated bile ducts were variable. All of the animals with jaundice and dilated bile ducts showed hypercholesteremia and most of the values for the esterified cholesterol were within the normal range or slightly above normal. The hypercholesteremia noted in these rats with obstructive jaundice is consistent with the results obtained in man (2) and the dog (7). Epstein (2) reported that in one-half of his obstructive jaundice cases, the cholesterol esters rose proportionately with the free cholesterol; in other cases, the ester fraction did not rise sufficiently to maintain the normal ratio, and in eight of the forty-three cases there was a fall in cholesterol esters. Hawkins and Wright (7) demonstrated that ligation of the common bile duct in the dog caused an increase in the absolute amount of cholesterol esters which paralleled the hypercholesteremia. The cholesterol ester concentration was reduced in these animals only after superimposing added damage by the administration of chloroform. The cholesterol esters in the blood of the jaundiced rat with ligated bile ducts did not parallel the free cholesterol, resulting in a lowered cholesterol ester ratio.

Sinclair (8) has reviewed the significance of the phospholipid to free cholesterol ratio. Data have been presented which show a high correlation of free cholesterol and phospholipid phosphorus over a wide range of concentrations. No adequate explanation can be given for this relationship.

SUMMARY

The concentration of blood plasma free and ester cholesterol and phospholipid phosphorus was determined at frequent intervals in

rats subjected to ligation of the bile duct and to partial hepatectomy.

A marked decrease in the cholesterol esters was noted on the 1st day after partial hepatectomy. This was assumed to be due to marked damage of the liver remnant. The esters returned to normal on the 2nd and 3rd days. The free cholesterol concentration was increased on the 2nd day after operation.

In the animals with ligated bile ducts, there was an increase in the concentration of free cholesterol. The esters were within the normal range or slightly above normal.

The phospholipid phosphorus varied directly with the free cholesterol concentrations in the animals subjected to both partial hepatectomy and ligation of the bile duct.

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THE RESOLUTION OF *dl*-LYSINE*

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(Received for publication, May 4, 1936)

Several procedures for the preparation of *dl*-lysine are recorded in the literature, among the synthetic methods the recent relatively simple and inexpensive one of Eck and Marvel (7). The present communication describes the separation of *dl*-lysine into its optical components. The procedure provides the only step still necessary to complete the manufacture of natural dextro-rotatory *l*(+)-lysine¹ entirely from a non-protein source. It also renders available for the first time the unnatural levorotatory *d*(-) modification, of interest to us for use in comparative growth studies.

EXPERIMENTAL

We have resolved *dl*-lysine by fractionally crystallizing its camphorates from 50 per cent methyl alcohol. The *l*(+)-lysine compound with *d*-camphoric acid is the less soluble and hence the more readily purified; with *l*-camphoric acid the reverse is true. The lysine isomers are readily isolated from the camphorates as the dihydrochlorides.

Preparation of d- and l-Camphoric Acids—The *d*-camphoric acid was obtained by oxidizing refined *d*-camphor with nitric acid, as directed by Noyes (13). Unchanged camphor was removed and the crude camphoric acid was purified through the anhydride, essentially as outlined by Aschan (1, a, b). From

* This communication was presented in abstract before the American Society of Biological Chemists at Washington, March 26, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, viii (1936); *J. Biol. Chem.*, **114** (1936)).

¹ Since natural lysine belongs to the *l* series of amino acids (Karrer, Escher, and Widmer (10); Lutz and Jirgensons (12)), but is dextrorotatory, specific designation by both letter and sign as suggested by Freudenberg and Rhino (8) for similar cases seems less confusing.

100 gm. of *d*-camphor, 70 to 85 gm. of crude *d*-camphoric acid (53 to 64 per cent of the theoretical) and 50 to 65 gm. of the purified product (38 to 49 per cent) were readily obtained. Physical constants appear in Table I.

The *l*-camphoric acid was prepared from *l*-borneol,² a source recommended by Campbell (5) as being more satisfactory than *l*-camphor. The method used was essentially identical with that outlined above for the preparation of *d*-camphoric acid from *d*-camphor. From 100 gm. of *l*-borneol (used as purchased, with-

TABLE I

Physical Constants of d-Camphor, l-Borneol, and d- and l-Camphoric Acids

Compound	M.p. (corrected)		[α] _D ²⁰ in absolute alcohol solution*	
	Observed	Reference	Observed	Reference
	°C.	°C.	degrees	degrees
<i>d</i> -Camphor†	178	178.6 (14)	+44.64	+44.20 (14)‡
<i>l</i> -Borneol†	206-207	208-209 (9)	-35.85	-37.74 (2)
<i>d</i> -Camphoric acid	188-189	188.2 (14)	+48.45	+47.75 (14)§
		187 (1, c)		+49.7 (1, c)
<i>l</i> -Camphoric "	188-189	187.5 (14)	-48.47	-48.12 (14)‡

The figures in parentheses denote bibliographic reference numbers.

* The concentration was that used in the reference cited. All readings by the author were at 20°.

† The camphor and borneol used by us were as purchased; the references are to purified preparations.

‡ Read at 16°.

§ Read at 17°.

|| Read at 16.5°; white light used.

out purification) 73 gm. of crude *l*-camphoric acid (56.2 per cent) and 42 gm. of the highly purified product (32.3 per cent) were readily obtained. Physical constants of the borneol and the camphoric acid are recorded in Table I.

Preparation of dl-Lysine Dihydrochloride—The synthesis of Eck and Marvel (7) was used with only minor changes. It seemed more convenient to prepare the cyclohexanoneoxime according to the method of Bousquet (4), suggested by Eck and Marvel as an alternative procedure. Acetone was found more satisfactory than

* Obtained from The British Drug Houses, Ltd., London.

ether for the precipitation of lysine dihydrochloride from the alcohol solution of its syrup. When ether is used, the lysine dihydrochloride becomes gummy unless the syrup is sufficiently concentrated. Acetone prevents this because it also dehydrates. Physical properties and analyses may be found in Table III. The over-all yield and, with but one exception, also the yields at each step of the synthesis were as good as those claimed by the authors of the method.

Preparation of Lysine Camphorates—To 76.7 gm. of *dl*-lysine dihydrochloride (0.35 mole) dissolved in approximately 200 cc. of water was added slightly over 0.35 mole of freshly prepared silver oxide. The mixture was shaken to facilitate the precipitation of the chloride, the latter was filtered off, and excess silver was removed from the filtrate as the sulfide. The filtrate was partially concentrated *in vacuo*, 35.0 gm. of *d*-camphoric acid (0.175 mole) dissolved in methyl alcohol were added, and the concentration was carried to dryness. The residue showed $[\alpha]_D^{20} = +7.94^\circ$.³

The mixture of *l*(+)- and *d*(-)-lysine *d*-camphorates was recrystallized from a minimal volume of 1:1 methyl alcohol-water solution. The mother liquors were concentrated to incipient crystallization, whereupon an equal volume of methyl alcohol was added. This process was repeated as long as fractions of appreciable size were obtained. Usually each crystallization was allowed to continue overnight in the cold. Each of the more insoluble fractions was similarly refractionated. Such a procedure must necessarily be followed closely by optical measurements, since practically unavoidable variations in technique influence the course of the fractionation. The following is a brief outline of the progress of one specific separation. Fraction I, 8.6 gm., showed $[\alpha]_D^{20} = +15.33^\circ$; Fraction II, 28.5 gm., $[\alpha]_D^{20} = +13.93^\circ$; Fraction III, 48.7 gm., $[\alpha]_D^{20} = +3.2^\circ$. On recrystallization from 50 per cent methyl alcohol, Fraction I yielded 8.1 gm., $[\alpha]_D^{20} = +16.14^\circ$, and Fraction II, 20.4 gm., $[\alpha]_D^{20} = +16.35^\circ$. These recrystallized fractions were combined and again recrystallized, yielding 22.53 gm. of optically pure, $[\alpha]_D^{20} = +16.41^\circ$, *l*(+)-lysine *d*-camphorate, or 52.3 per cent of the theoretical amount expected from 76.7 gm. of *dl*-lysine dihydrochloride. Further recrystalliza-

³ Aqueous solutions of the camphorates (*c* = 2.00 gm. per 100 cc.) were always employed

tion did not change the rotation. Physical and chemical constants of this product are recorded in Table II.

Further fractionation of mixtures showing rotations of $+4.5^\circ$ or less proved too laborious to be practical. Consequently Fraction III and the dry residues from the various mother liquors were dissolved in water and made acid to Congo red with sulfuric acid. The *d*-camphoric acid was extracted with ether and barium hydroxide was cautiously added to effect the exact removal of the sulfate ion. The calculated amount of *l*-camphoric acid needed to replace the *d*-camphoric acid removed was next added and the solution was concentrated to dryness. Fractionation of the *l*-camphorate mixture, essentially as outlined for the *d*-camphorate, yielded 27.79 gm. of *d*(-)-lysine *l*-camphorate (64.5 per cent of

TABLE II
Melting Points and Analyses of the Lysine Camphorates

Compound	M. p. (corrected)	Nitrogen found*	$[\alpha]_D^{20}$, solution in water; $c = 2.00$
	$^\circ\text{C.}$	per cent	degrees
<i>l</i> (+)-Lysine <i>d</i> -camphorate.....	245-246	11.25	+16.41
“ <i>l</i> -camphorate.....	239-240	11.35	+ 0.35
<i>d</i> (-)-Lysine “.....	245-246	11.30	-16.39
“ <i>d</i> -camphorate.....	239-240	11.24	-0.38

* Calculated for lysine camphorate, $(\text{C}_6\text{H}_{14}\text{O}_2\text{N}_2)_2 \cdot \text{C}_8\text{H}_{14}(\text{COOH})_2$, 11.38 per cent.

the theoretical amount expected from the *dl*-lysine dihydrochloride used) which showed $[\alpha]_D^{20} = -16.39^\circ$. The physical and chemical constants determined appear in Table II.

On removing *l*-camphoric acid from the lower rotating *l*-camphorate fractions and substituting *d*-camphoric acid, 11.62 gm. more of optically pure *l*(+)-lysine *d*-camphorate were obtained, bringing the total yield to 34.15 gm. (79.3 per cent). A second substitution of *l*-camphoric acid instead of *d*- in this second set of low rotating *d*-camphorate fractions made possible the isolation of 7.00 gm. more of optically pure *d*(-)-lysine *l*-camphorate, increasing the yield to 34.79 gm. (80.8 per cent). Further fractionation in this way, though impractical, would undoubtedly increase these yields.

Of value for reference are the properties of *l*(+)-lysine *l*-camphorate and *d*(-)-lysine *d*-camphorate prepared from the free lysines and the purified camphoric acids. These are given in Table II.

Isolation of l(+)- and *d*(-)-Lysine As the Dihydrochlorides—To 19.7 gm. of *l*(+)-lysine *d*-camphorate (0.04 mole) dissolved in 100 to 200 cc. of water were added 20 cc. of concentrated hydrochloric acid. The bulk of the camphoric acid liberated was filtered off and the residue was extracted with ether. The hydrochloric acid-lysine solution was concentrated *in vacuo* to a thin

TABLE III

Melting Points and Analyses of the dl-, l(+)-, and *d*(-)-Lysine Dihydrochlorides

Lysine dihydrochloride	M.p. (corrected)	Nitrogen found*	$[\alpha]_D^{20}$, solution in water
	*C.	per cent	degrees
<i>dl</i>	188–190†	12.76	0.0
<i>l</i> (+)	201–202‡	12.78	+15.63 (<i>c</i> = 3.00)§ +16.55 (" = 16.00)
<i>d</i> (-)	201–202	12.73	-15.65 (" = 3.00)

* Calculated for $C_6H_{14}O_2N_2 \cdot 2HCl$, 12.79 per cent.

† 188–190°, Eck and Marvel (7).

‡ 200–201°, Cox, King, and Berg (6).

§ +15.57°, in water, *c* = 2.86, Lawrow (11); +15.4°, in water, *c* = 4.38, Lutz and Jirgensons (12).

|| +16.44°, in water, *c* = 16.27, Bergmann and Zervas (3); +16.36° and 16.68°, in water, for *c* = 11.43 and *c* = 19.48 respectively, Lawrow (11).

syrup which was warmed on the steam bath and dissolved in a minimum amount of alcohol. 3 to 5 volumes of acetone were added to precipitate the lysine dihydrochloride. This was redissolved in alcohol and again precipitated with acetone. 17.1 gm. of *l*(+)-lysine dihydrochloride (97.6 per cent of the calculated amount) were thus isolated. The constants found appear in Table III.

19.7 gm. of *d*(-)-lysine *l*-camphorate, similarly treated, yielded 17.16 gm. (97.9 per cent of the calculated amount) of *d*(-)-lysine dihydrochloride. The properties of this product are also recorded in Table III.

The yields of pure camphorate and pure lysine dihydrochloride indicate that 77.4 per cent of the *l*(+)-lysine dihydrochloride and 79.1 per cent of the *d*(-)-lysine dihydrochloride can be separated from the racemic mixture by this procedure. More intensive fractionation would increase this yield.

DISCUSSION

The method of resolution described is simple and direct. The camphoric acids are easily prepared from relatively cheap sources. Their purification through the anhydrides is not difficult, but does cause considerable loss in yield. This is probably not essential, since good separations of *l*(+)-lysine have been secured with *d*-camphoric acid having a specific rotation of $+44.6^\circ$. *d*-Camphoric acid of this grade can be obtained on the market.

Even when molecularly equivalent amounts of *dl*-lysine and *d*-camphoric acid were used, the compound yielded by recrystallization contained 1 molecule of camphoric acid to 2 of lysine.

Several modifications in fractionation procedure are possible. Thus when only the natural *l*(+)-lysine dihydrochloride is required, its yield may be increased by racemizing the lysine in the more soluble *d*-camphorate fractions to provide more *dl*-lysine. To secure *l*(+)-lysine alone, the use of *d*-camphoric acid only is necessary; for the isolation of *d*(-)-lysine, only *l*-camphoric acid is required. If *l*-camphoric acid is not available, a fair yield of *d*(-)-lysine should be obtainable from the more soluble *d*-camphorate fraction with *dl*-camphoric acid.

SUMMARY

The resolution of *dl*-lysine dihydrochloride has been accomplished by the use of *d*- and *l*-camphoric acids. Fractional crystallization of the *d*-camphorates readily yields the less soluble *l*(+)-lysine *d*-camphorate; of the *l*-camphorates, the *d*(-)-lysine *l*-camphorate. By the alternate use of *d*- and *l*-camphoric acids, yields of 77 per cent or better of each of the pure optical components of *dl*-lysine dihydrochloride have been obtained.

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β -CHOLESTEROL*

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(Received for publication, May 7, 1936)

In 1908 Diels and Linn (1), by the reduction of cholestenone with metallic sodium, obtained a small amount of a crystalline and apparently homogeneous substance melting at 160° , which they regarded as an isomer of cholesterol and to which they assigned the name of β -cholesterol. In 1932, Wagner-Jauregg and Werner (2) demonstrated that the product was, in reality, a molecular compound of two sterols of which one was precipitable by digitonin. They found this component to be dihydrocholesterol. The properties of the molecular compound indicated that the non-precipitable sterol must be an unsaturated alcohol, isomeric with cholesterol, although they were unsuccessful in isolating any homogeneous product from the non-precipitable portion of the molecular compound.

In repeating this work, we have found that the non-precipitable portion of β -cholesterol gives a positive Rosenheim test and is quantitatively dehydrated on treatment with acid to Δ -2,4-cholestadiene (3); it has proved to be identical with the epiallocholesterol recently obtained by us from the reduction of cholestenone with aluminum isopropylate (3).

The recrystallization of a mixture of equimolar amounts of dihydrocholesterol and epiallocholesterol from acetone gives a molecular compound crystallizing in the shining needles characteristic of β -cholesterol. The rotation ($+72^{\circ}$ in benzene) is

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† Columbia University Fellow, 1934-36. This report is from a dissertation submitted by E. A. Evans, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

similar to that reported for β -cholesterol (+69°). The melting point of the synthetic β -cholesterol, 154°, shows no change on repeated crystallization. The higher melting point, 160°, of the Diels and Linn material may be due to the presence of the high melting cholesterol-pinacone, since we have found small quantities of this compound in β -cholesterol prepared by the reduction of cholestenone with metallic sodium.

EXPERIMENTAL

A solution of 5.5 gm. of digitonin in 500 cc. of 95 per cent ethyl alcohol was added to a solution of 2.95 gm. of β -cholesterol (prepared according to Wagner-Jauregg and Werner (2)) in 350 cc. of alcohol. After 24 hours the precipitate was filtered off, washed, and extracted with dry ether in the Soxhlet apparatus for 1 hour. The filtrate, together with the ether extract, was taken to dryness *in vacuo* and the residue repeatedly extracted with dry ether. The ether solutions were taken to dryness and the light yellow oil remaining was dissolved in 60 cc. of acetone. On standing in the ice chest, a small amount of cholesterol-pinacone, m.p. 221°,¹ settled out after 48 hours. By the cautious addition of water to the acetone solution, 1.1 gm. of crystalline material were obtained, m.p. 70–75°. Recrystallization of this material from acetone in the cold gave pure epiallocholesterol, m.p. 84°, $[\alpha]_D^{22} = +118.2^\circ$ (1 per cent in benzene). The mixed melting point with epiallocholesterol showed no depression. For further identification, 30 mg. of the compound were refluxed with alcoholic HCl for 2 hours, when Δ -2,4-cholestadiene was obtained in quantitative yield (3).

SUMMARY

The β -cholesterol of Diels and Linn is a molecular compound of 1 mole of dihydrocholesterol with 1 mole of epiallocholesterol.

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¹ All melting points are corrected.

THE PREPARATION OF LITHOCHOLIC ACID FROM CHOLESTEROL*

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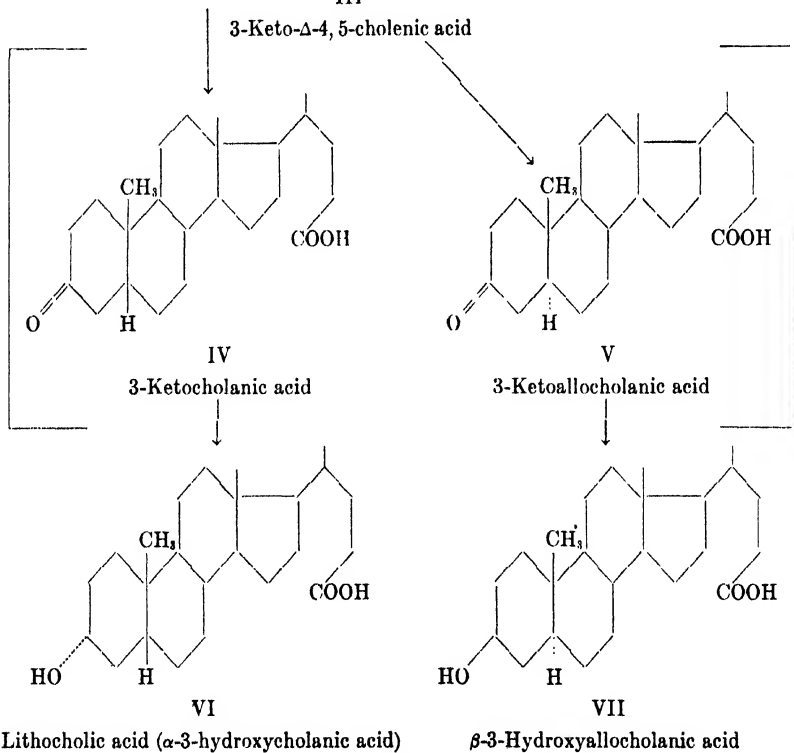
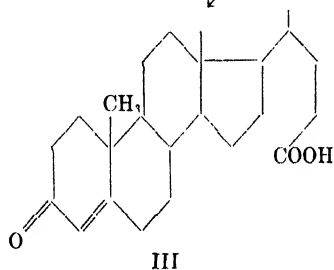
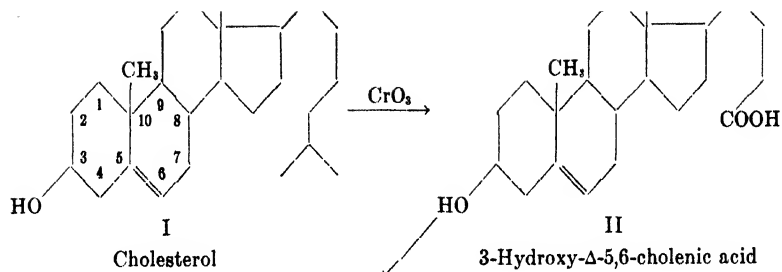
None of the natural bile acids has as yet been prepared from cholesterol. In the methods available for the preparation of lithocholic acid (α -3-hydroxycholanic acid), either cholic acid (1) or desoxycholic acid (2) is the starting material.

For metabolic studies it was desirable to have a procedure by which deuterium could be introduced into lithocholic acid. A convenient method for the introduction of this isotope into organic molecules is catalytic hydrogenation with gaseous deuterium (3). As the known methods for the preparation of lithocholic acid start with the use of saturated compounds, they are not suitable for our purposes. The new procedure with the unsaturated cholesterol as a starting material involves catalytic hydrogenation and can therefore also be used for the preparation of deuterium-containing lithocholic acid.

The side chain of lithocholic acid (VI), like that of all other bile acids, has 3 carbon atoms less than that of cholesterol. The steric configuration of the ring skeleton and of the hydroxyl group at carbon atom 3 has been established by Windaus (4) and by Ruzicka and Goldberg (5). The hydrogen atom at carbon atom 5 is in *cis* position to the methyl group at carbon atom 10; *i.e.*, the ring system corresponds to that of coprostane (*cis*-decalin). The hydroxyl group at carbon atom 3 is *trans* to the methyl group at carbon atom 10. Lithocholic acid, therefore, is that bile acid which corresponds to epicoprosterol.

The side chain of the sterols ($C_{27}H_{47}$) may be oxidized to the side

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.



chain of the bile acids ($C_{27}H_{46}O_2$) by chromic acid (6). On oxidation of cholesterol dibromide acetate the dibromide acetate of 3-hydroxy- $\Delta^5,6$ -cholenic acid is formed, from which on debromination and saponification the free unsaturated acid (II) can be obtained. This acid has been isolated from the mother liquor of the preparation of dehydroandrosterone (7).¹ The ring skeleton and the position of the hydroxyl group and of the double bond in this acid correspond to cholesterol. The preparation of lithocholic acid from this compound is similar to the preparation of epicooprosterol from cholesterol (8).

As the acid is difficultly soluble in organic solvents, the methyl ester was used for the work. On bromination, oxidation with chromic acid, and debromination the methyl ester of the 3-keto- $\Delta^4,5$ -cholenic acid (III) was obtained in good yield. This ester could be saponified at room temperature. The free acid so obtained corresponds to cholestenone and has the same absorption spectrum, indicating that the double bond is in α,β position to the keto group (9).

On hydrogenation of the ester with platinum oxide 2 moles of hydrogen are taken up. The product consists of a mixture of about 80 per cent lithocholic acid ester (VI) and 20 per cent of the ester of β -3-hydroxyallocholanolic acid (VII). The two esters can be separated from each other quantitatively by the aid of digitonin, as has been shown by Fernholz (10) in the case of the corresponding acids.

The formation of β -3-hydroxyallocholanolic acid, the constitution of which corresponds to dihydrocholesterol, can be explained by the following mechanism: on hydrogenation of the unsaturated keto acid the double bond in the ring is saturated first, as in the case of cholestenone. On partial hydrogenation of the unsaturated keto acid with active palladium, there was obtained instead of pure ketocholanolic acid (IV) a mixture of this acid with a small amount of ketoallocholanolic acid (V). This mixture can be separated only with difficulty. The saturation of the double bond, therefore, leads to derivatives of *cis*-, as well as *trans*-decalin. On

¹ The authors are indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for placing considerable amounts of insoluble sodium salts, obtained from the large scale preparation of dehydroandrosterone, at their disposal.

subsequent hydrogenation of the keto group at neutral reaction the hydroxyl groups in both acids are formed in *trans* position to the hydrogen atom at carbon atom 5—a new indication that on hydrogenation of the keto group at carbon atom 3 the position of the hydrogen atom at 5 governs the steric configuration of the resulting carbinol (8).

The yield of analytically pure lithocholic acid was about 36 per cent, calculated for 3-hydroxycholenic acid.

EXPERIMENTAL

3-Keto-Δ-4,5-Cholenic Acid Methyl Ester—To 4.19 gm. of 3-hydroxy-Δ-5,6-cholenic acid methyl ester (7), m.p. 144°, in 50 cc. of acetic acid were slowly added, with cooling, 1.73 gm. of bromine (1 M) in 18.8 cc. of acetic acid. After the bromine was taken up, 1.1 gm. of CrO_3 (1.5 M) in 20 cc. of acetic acid and 5 cc. of water were added. After 20 hours at room temperature, water was added and the mixture extracted with ether. The ether was washed several times with water. For the debromination (11) an excess of sodium iodide in methyl alcohol was added, the ether and part of the methyl alcohol were distilled off, and the deep brownish solution was refluxed for 2 hours. The solution was distributed between ether and an aqueous solution of sodium sulfite. The ether solution, after standing over Na_2SO_4 , was distilled off and the residue recrystallized from methyl alcohol. Yield 2.3 gm. or 55 per cent of the theory; m.p. 125°. Heavy colorless prisms, easily soluble in ether, chloroform, and carbon tetrachloride and less soluble in ethyl and methyl alcohols, were obtained.

$\text{C}_{26}\text{H}_{44}\text{O}_2$. Calculated, C 77.72, H 9.84; found, C 77.57, H 9.56

3-Keto-Δ-4,5-Cholenic Acid—150 mg. of the ester were dissolved in 7 cc. of methyl alcohol and 1 gm. of KOH in 7 cc. of methyl alcohol was added. After 2½ hours standing at room temperature, water was added and the solution was acidified with H_2SO_4 and extracted with ether. The residue from the ether was recrystallized three times from aqueous acetone. Small prisms, m.p. 185–186°, easily soluble in acetone and alcohol and slightly

soluble in ether, were obtained. The substance shows a strong absorption at $240\text{ m}\mu$ (Fig. 1).²

$\text{C}_{24}\text{H}_{40}\text{O}_5$. Calculated, C 77.42, H 9.77; found, C 77.12, H 9.77

Semicarbazide of 3-Keto- Δ -4,5-Cholenic Acid Methyl Ester—45 mg. of the ester were refluxed for 1 hour with 100 mg. of semicarbazone acetate in alcohol. The gelatinous precipitate formed on cooling was dissolved in hot alcohol, and water was added. Long needles formed which were again recrystallized. M.p. 229° under decomposition.

$\text{C}_{28}\text{H}_{40}\text{O}_5\text{N}_2$. Calculated, N 9.48; found, N 9.31

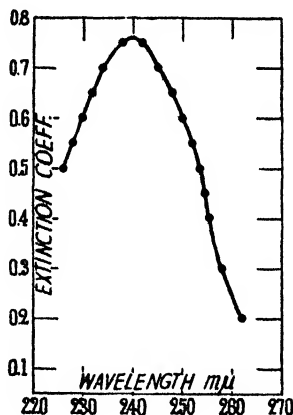


FIG. 1. 3-Keto- Δ -4,5-cholenic acid, 1.54 mg. in 100 cc. of absolute ethyl alcohol; 2 cm. cell; 21° .

Lithocholic Acid—2.2 gm. of 3-keto- Δ -4,5-cholenic acid methyl ester were shaken in 20 cc. of alcohol and 20 cc. of ether with 300 mg. of platinum oxide in an atmosphere of hydrogen. 2 moles of hydrogen were taken up within 30 minutes. The reduction product was dissolved in ethyl alcohol and an excess of digitonin in 80 per cent alcohol added. 2.1 gm. of insoluble digitonide were obtained after 15 hours standing. The mother liquor from the digitonin precipitation was brought to dryness, extracted with

² The authors are indebted to Dr. H. Darby for making the absorption spectrum readings.

ether, the ether solution concentrated to a small volume, and a 7 per cent solution of KOH in methyl alcohol added. After standing overnight, water was added and the mixture was acidified with sulfuric acid and extracted with ether. The residue from the ether was recrystallized from acetone. 1.41 gm. (yield 66 per cent); m.p. 186–187°; $[\alpha]_D^{24} = +32.56^\circ$ in absolute alcohol. No depression of the melting point with an authentic preparation of lithocholic acid³ was observed.

β -3-Hydroxyallocholanolic Acid—The digitonide obtained from the reduction product of 3-keto- Δ -4,5-cholenic acid was split with pyridine and ether in the manner described for sterols (12). The residue from the pyridine-ether mixture was saponified as described for lithocholic acid and the resulting acid recrystallized from aqueous alcohol. 0.31 gm.; m.p. 220–221°. No depression of the melting point when mixed with β -3-hydroxyallocholanolic acid obtained from the oxidation of dihydrocholesterol acetate (13) was observed.

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³ The authors are indebted to Mr. Paul de Haen for supplying them with a sample of pure lithocholic acid for comparison.

A NEW METHOD FOR THE DETERMINATION OF METHIONINE IN PROTEINS

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(Received for publication, April 16, 1936)

The only method which has thus far been published for the determination of methionine in proteins depends on the recovery of volatile iodide (1) when purified proteins are boiled with 57 per cent hydriodic acid. The sulfur equivalent of this fraction, when added to the sulfur equivalent of the cystine fraction (as determined iodometrically in HCl digests after reduction with zinc), accounts for all of the sulfur in a large number of proteins (2).

In some cases high recoveries of total sulfur indicated that one or both methods were giving incorrect results. There was a strong probability that volatile iodide was arising from sources other than methionine, such as glycerol from fat, alcohol, and ether from purification procedures, and carbohydrate. Barritt (3) found, however, that the original method gave satisfactory results when applied to wool. The results were not higher than was expected and did not materially alter the conclusion that practically all the sulfur of this protein is cystine sulfur.

It was desirable, nevertheless, to attempt to devise a second method for comparison, which would not be subject to the errors mentioned above.

A study of the hydriodic acid digest showed that cystine had been reduced quantitatively to cysteine and could be determined iodometrically or by the Sullivan or Folin and Marenzi colorimetric methods (4). The question of the non-volatile residue from methionine was solved when the thiolactone of homocysteine was isolated.

This conversion has recently been confirmed by Riegel and du Vigneaud (5), who also studied the conditions governing the opening and closing of the ring.

The present method is based on the determination of this non-volatile residue from methionine. Attempts to determine it directly were unsuccessful, and therefore an indirect method was devised. The chief difficulty was in finding an oxidant which would be specific for sulfhydryl under the conditions necessary for opening the thiolactone ring. The presence of cysteine in the protein digests placed additional limitations upon the available methods.

In acid solutions cysteine may be determined without interference from thiolactone. The Folin and Marenzi and Sullivan reactions have both been adapted to our hydriodic acid digests, but were found wanting in dependability. Since the Okuda method has been shown by Sullivan and Hess (6) to be practically as specific as Sullivan's naphthoquinone method and since our digests meet the requirements of high acidity and high iodide concentration, we have chosen this simple procedure for determining cysteine. Hydriodic acid digestion eliminates the necessity of decolorization and zinc reduction. Direct titration is not feasible owing to the slowness of the iodine oxidation near the equivalence point and therefore we add a small excess of iodate and titrate back with thiosulfate, with starch as indicator.

Thiolactone cannot be quantitatively determined by opening the ring with alkali and then acidifying and titrating with iodate. The ring closes too rapidly and there is significant oxidation of sulfhydryl by the air when the solution is alkaline. Besides, when cysteine has first been determined as described above, the opening of the thiolactone ring by alkali is followed by oxidation of homocysteine by the tetrathionate. If sufficient tetrathionate is present and air is excluded, the reaction is quantitative, yielding an equivalent quantity of thiosulfate which may be titrated with iodate after acidification. This reaction has therefore been utilized for the determination of thiolactone derived from methionine in proteins.

The qualitative proof that tetrathionate oxidizes homocysteine to homocystine was obtained as follows: About 10.0 mg. of thiolactone dissolved in 2.0 cc. of HI were made alkaline with NH_4OH , a crystal of sodium nitroprusside was introduced, and sodium tetrathionate solution was added until the purple color disappeared. Addition of cyanide brought the color back. At-

tempts to crystallize out the homocystine were unsuccessful owing to the small amounts of material available. A repetition of the above experiment with about 1.0 gm. of cysteine gave on neutralization a copious precipitate of cystine. The filtrate gave three reactions for thiosulfate. On acidification sulfur and SO_2 were evolved and iodine was reduced. AgNO_3 gave a yellow precipitate which quickly turned black. These facts are in agreement with older observations that alkali sulfides reduce tetrathionate to thiosulfate.

The specificity of the reaction was tested on a mixture of amino acids of the following composition: methionine, cystine, glycine, alanine, isoleucine, phenylalanine, tyrosine, tryptophane, arginine,

TABLE I
Recovery of 10.0 Mg. Each of Cystine and Methionine from Mixture of Amino Acids and Glucose

Cystine	Methionine	
	Volatile iodide	Homocysteine
mg.	mg.	mg.
10.06	10.01	10.10
10.26	10.00	10.00
10.30	9.46	9.84
9.98	10.30	9.92
Average . . 10.15	9.94	9.96

valine, aspartic acid, glutamic acid, histidine, and glucose. These were dissolved in hydriodic acid so that 10.0 cc. contained 10.0 mg. of each of the substances present. This mixture was put through the procedures to be described for analyzing proteins with the results shown in Table I.

The recoveries of cystine and methionine are entirely satisfactory and prove that the small amounts of hypophosphite present do not interfere with the measurement of iodine consumption by sulfhydryl. The hypophosphite originally present in the hydriodic acid has, of course, been decomposed during the heating, yielding phosphine, which was absorbed in the saturated mercuric chloride. Tetrathionate oxidation is specific in the presence of these protein constituents and glucose does not yield volatile iodide.

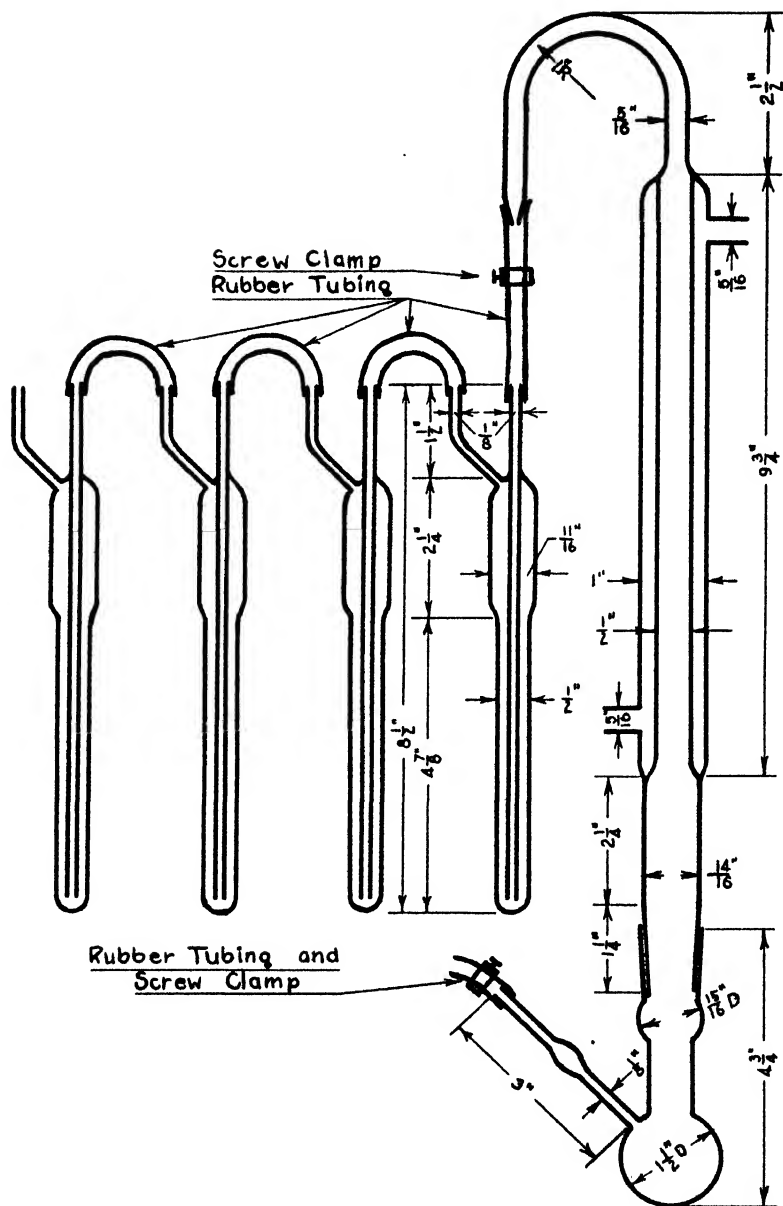


FIG. 1. Apparatus for determination of sulfur distribution in proteins

We have been unable to find a sample of natural leucine which did not contain at least 5.0 per cent methionine.

We have analyzed two commercial preparations and one from our own laboratory and all contained methionine (5 to 20 per cent).

Procedures

The apparatus is the same as has been used in previous studies and is shown in Fig. 1.

The proteins for analysis are extracted for 24 hours with petroleum ether and dried in a vacuum desiccator over P_2O_5 . The use of ethyl ether should be avoided, since incomplete removal of the last traces increases volatile iodide. Several samples of proteins dried at 105° turned slightly brown and on analysis showed low cystine and methionine content.

Hydrolysis of Proteins—About 0.5 gm. of protein is weighed and transferred to the digestion flask. 10.0 cc. of 57 per cent HI containing 1.0 per cent KH_2PO_4 and a small boiling chip are added and the flask connected to the condenser kept at $50-60^\circ$. The absorption train consists of four scrubbers: Scrubber 1, 20 per cent $CdCl_2$ with 20 per cent $BaCl_2$; Scrubber 2, saturated $HgCl_2$; Scrubbers 3 and 4, glacial acetic acid with 10.0 per cent potassium acetate and bromine (6 drops per 10.0 cc.).

It has been found unnecessary to keep the scrubbers warm. The $BaCl_2$ has been added to Scrubber 1 to catch any SO_2 which might escape reduction to H_2S . Nitrogen has been substituted for CO_2 for aeration. With the upper pinch-cock open and the lower one closed, the microburner is lit. Bubbling begins at once, owing to expansion of the air. When this has stopped and the flasks are boiling quietly and the iodine first formed is entirely reduced by hypophosphite, the lower cock admitting nitrogen is opened and the upper one adjusted to maintain a constant bubbling rate such that the bubbles can be counted. Boiling and aeration are continued for 6 hours. The scrubbers are disconnected and the condenser jacket drained. Connection is then made to a second vertical condenser kept cold and the digest is concentrated to about 3.0 cc. Care must be exercised that the digest is not burned as it concentrates.

Dilution of Digest—The flask containing the digested protein is disconnected, two or three crystals of KH_2PO_4 are added, and the

mixture is boiled for about 1 minute to remove any iodine. It is then rinsed into a 25.0 cc. volumetric flask with 4 per cent HCl which has been saturated with nitrogen. The flask is tightly stoppered, cooled under the tap, and made up to volume.

Analysis of Digest—Two 10.0 cc. samples are measured into 50.0 cc. Erlenmeyer flasks and deaerated at the water pump. The first flask is disconnected and a small excess of 0.02 N potassium biiodate added. (2.0 cc. are required for every 10.0 mg. of cystine present in 25.0 cc. of the digest.) A few drops of starch solution are added and the excess iodine titrated with 0.02 N thiosulfate. A blank digest without protein is treated similarly to determine available iodine. From the iodine consumed the quantity of cystine present can be calculated.¹ 2.0 cc. of sodium tetrathionate are added and the flask returned to the pump for deaeration. The sodium tetrathionate is prepared from 0.1 N biiodate and 0.1 N thiosulfate with the aid of a little KI and HCl. The connection between the pump and the flask is made through a 3-way glass stop-cock and rubber stopper. When the air is removed from the digest, a burette containing concentrated ammonium hydroxide is connected to the side tube of the stop-cock and 3.0 cc. are allowed to be drawn in. The flask is then evacuated and closed off. Care must be exercised, as the digest foams badly. After 15 minutes the flask is removed, acidified with 10.0 cc. of 10 per cent HCl, and titrated with 0.02 N biiodate. Methionine is calculated from the quantity of iodine consumed by thiosulfate which was formed in the reduction of tetrathionate by homocysteine. It is desirable to have only a small excess of tetrathionate and a minimum of ammonium hydroxide or a considerable blank will result from decomposition of tetrathionate by alkali. Sodium hydroxide cannot be substituted for ammonium hydroxide.

Determination of Volatile Iodide—Scrubbers 3 and 4 are rinsed into a 100 cc. volumetric flask containing 5.0 cc. of 25 per cent sodium acetate. A small excess of formic acid (sp. gr. 1.20) is added to reduce excess bromine and the flasks are whirled and diluted to the mark. 25.0 cc. samples are added to a little KI and a few drops of 10 per cent H₂SO₄ and the iodine titrated with 0.02 N thiosulfate. Methionine is calculated from the iodine equivalent of the thiosulfate used.

¹ These results are reported in the following paper.

Results

Table II shows the comparative figures by the two methods for methionine. The last column gives the per cent recovery by the new method compared with the old. The discrepancy is probably explained by the fact that for every molecule of volatile iodide obtained 6 atoms of iodine are titrated. This results in a tendency for the values obtained by the old method to be high. When one considers the possibility of HI, I₂, and other volatile iodides being absorbed, the agreement is remarkable. In view of the results to

TABLE II
Methionine Content of Proteins

Protein	Source	Per cent methionine		Homocysteine Volatile iodide $\times 100$
		Volatile iodide	Homo- cys- teine	
Lactalbumin.....	Harris	2.45	2.32	94.7
Casein.....	"	3.31	3.10	93.6
Edestin.....	Hemp	2.38	2.20	92.5
Ovalbumin.....	Egg	5.07	4.49	88.5
Livetin.....	Jukes	2.33	2.35	100.7
Ovomucoid.....	"	1.43	1.38	96.4
Vitellin.....	"	2.70	2.60	96.3
α -Globulin, adzuki bean.....	Jones	2.09	2.02	96.7
Zein.....	Harris	2.58	2.46	95.4
".....	Jones	2.32	2.25	97.0
Albumin.....	Merek	3.84	3.55	92.0
Peptone.....	Witte	2.19	2.20	100.5
Edestin.....	Pearson	1.35	1.45	107.0
Average.				96.3

be reported in the following paper, we believe the new figures for methionine are nearer the true values.

We wish to thank Dr. T. H. Jukes and Dr. D. B. Jones for samples of proteins.

SUMMARY

1. A new method for methionine determination in proteins is described based on demethylation of methionine and oxidation of the resulting homocysteine with sodium tetrathionate.

2. When cysteine is also present, it must be determined first preferably by iodine oxidation.

3. The volatile iodide method yields satisfactory results provided ether, alcohol, and fat have been completely removed and provided the protein has not been burned in drying.

4. Glucose and presumably other carbohydrates do not yield volatile iodide under the conditions prescribed.

5. Small amounts of hypophosphite remaining in the digest do not interfere with iodine titrations carried out promptly at room temperature.

6. Tetrathionate oxidation of sulfhydryl is specific when tested in the presence of a limited number of amino acids and is not influenced by the factors which tend to give high results by the volatile iodide method.

7. Leucine preparations on the market are often contaminated with considerable methionine.

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THE SULFUR DISTRIBUTION IN PROTEINS

II. THE COMBINED METHODS FOR THE DETERMINATION OF CYSTINE, METHIONINE, AND SULFATES IN HYDRIODIC ACID DIGESTS

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In the first study of this problem (1) we were concerned primarily with the cystine determination. It seemed to us highly probable that the figures given by Sullivan and Hess (2) for cystine in proteins were too low. This could be due chiefly to the loss entailed by humin formation and removal. In addition, owing to the high specificity of the naphthoquinone method, any slight modification of the cystine molecule, incomplete digestion of the protein, or incomplete "reduction" by cyanide would lead likewise to low results. Since our first study was published, others have pointed out defects in the Sullivan method leading to low recoveries. Unpublished experiments from our own laboratory showed that even large amounts of non-cystine amino acids, when present with small amounts of cystine, resulted in competition for the color reagent and led to low recoveries.

We therefore devised the gasometric method for cystine (3) on hydrolysates from which the humin had not been removed. The results were only partially successful, for, as Hess pointed out (4) and as we also suspected, the humin was consuming some iodine.

A new possibility was opened up by the observation that hydriodic acid digests contained no humin but did contain cysteine. We therefore adapted the Sullivan method to these digests and found, in general, higher amounts of cystine than were reported for these proteins. No degree of confidence, however, could be placed on the results because of the variability obtained on apparently identical mixtures.

We therefore sought for a simpler and more dependable method for cystine, which would not interfere with the determination of the other sulfur fractions in the digests.

The advantages gained by the introduction of hydriodic acid as hydrolyzing agent were many and gave promise of the possibility of developing a system of analysis which was simple and inclusive of at least three fractions of sulfur. In addition to the quantitative reduction of cystine, methionine was demethylated and the volatile iodide could be recovered (5). Sulfates and other forms of oxidized sulfur were reduced to H_2S , which could also be separately absorbed and estimated.

The combination of the Okuda method for cystine, the volatile iodide method for methionine, and an iodometric method for H_2S gave excellent results. It was found, however, that extreme care in the preparation of the proteins was required to eliminate other sources of volatile iodide than methionine. During the reduction of sulfates to H_2S some SO_2 escaped and was lost.

Both of these problems were finally solved by the methods described in the preceding paper. A new method for methionine was developed on the basis of the non-volatile residue from methionine, which confirmed the results with the volatile iodide method on carefully purified proteins. The introduction of BaCl_2 in the CdCl_2 scrubber retained the small amounts of SO_2 which escaped reduction to H_2S .

The results here reported were obtained with these methods. It is difficult to decide between the volatile iodide method and the new tetrathionate oxidation of homocysteine. Perhaps one should run both methods and take an average. We have, however, selected the tetrathionate results for the present study in view of the ever present danger of high results by the volatile iodide method and the slight advantage gained by eliminating the analysis of the last two scrubbers in the absorption train. The train should be kept intact to prevent pollution of the air with phosphine and methyl iodide.

Procedures

The directions for preparing proteins, hydrolyzing, diluting the digest, and analysis for cysteine and homocysteine are given in the preceding paper.

Determination of H_2S and SO_2 —To the first scrubber, containing CdS and $BaSO_3$, are added an excess (usually 2.0 cc.) of 0.02 N biiodate and 2.0 cc. of a mixture of KI, HCl, and starch. The iodine liberated oxidizes CdS to S and $BaSO_3$ to $BaSO_4$. A blank determination gives available iodine minus the traces of iodine used to oxidize H_2S from the hydriodic acid. Recoveries averaged 98.8 per cent.

A few words of explanation of the blank determinations may be helpful. In this work one blank was run simultaneously with

TABLE I
Sulfur Distribution in Proteins

Protein	Source	Total S	Per cent total S			
			Cys- tine	Meth- ionine, homo- cys- teine	H_2S	Recov- ery
Casein.....	Harris	0.80	9.8	83.4	2.4	95.6
Lactalbumin.....	"	1.45	62.1	34.4	3.5	100.0
Zein.....	"	1.08	25.9	49.0	24.5	99.4
Edestin.....	Hemp	0.99	37.7	47.8	14.5	100.0
Albumin.....	Merck	1.64	46.0	46.5	2.8	95.3
".....	Egg	1.61	33.6	60.0	4.7	98.3
Peptone.....	Witte	0.89	44.5	53.1	1.0	98.6
Ovomucoid.....	Jukes	2.26	76.6	13.1	6.0	95.7
Vitellin.....	"	1.14	31.1	49.0	11.6	91.7
Livetin.....	"	1.80	55.2	28.0	8.4	93.6
Edestin.....	Pearson	0.62	43.9	50.3	3.6	97.8
Average.....						96.9

each set of three protein digests. 10.0 cc. of HI from the same stock as used for the proteins were placed in the digestion flask and treated exactly as a protein digest. From the analyses of the various scrubbers and contents of the flask the blank for each of the determinations is obtained.

Results

Table I gives the average figures obtained on eleven proteins with these new methods. Some differences will be noted from those previously published. In general, the cystine sulfur is

lower, owing to absence of humin in these digests. They still remain considerably higher than those reported by Sullivan. The methionine sulfur is also lower, since extraneous sources of volatile iodide are not here included. Some discrepancies occur owing to variations in the protein preparations.

The egg yolk proteins appear for the first time. The high value for cystine (6.50 per cent) in ovomucoid was not expected. Not much significance can be attached to the H_2S figures, since in all probability this fraction comes largely from inorganic sulfate used in the preparation of the sample and could be removed by dialysis. Some proteins like ovomucoid undoubtedly contain sulfate present as an ester, which is significant.

The average recovery of total sulfur (96.9 per cent) is satisfactory, considering the number of analyses necessary and the unavoidable errors of technique.

SUMMARY

1. A new system of analysis for the various fractions of sulfur is described. The methods are more specific and simpler than those previously used.

2. The most important single advance is the elimination of hydrochloric acid hydrolysis. The advantages of hydriodic acid over hydrochloric acid are more rapid hydrolysis, elimination of humin and reactive fragments of carbohydrate, reduction of cystine, demethylation of methionine with the formation of thiolactone, and reduction of sulfates. Only one hydrolysis is now required instead of two.

3. Only one carefully prepared standard solution is required; namely, 0.02 N potassium biiodate.

4. An average recovery of 96.9 per cent was obtained on the total sulfur of eleven proteins.

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THE EXTRACTION OF LIPIDS FROM THE RED BLOOD CELLS*

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(Received for publication, May 4, 1936)

In a previous communication (1) it has been shown that lipids may be completely extracted from blood plasma or serum without heat if diluted with 20 volumes or more of alcohol-ether. The maximum cold extraction of whole blood was found to require at least 30 volumes of solvent. No values for the red blood cells were reported at that time, because it was found that heat produced apparently higher values after maximum extraction had been obtained in the cold. A similar situation prevailed in extracts of whole blood. Since heating caused the extraction of colored products from the decomposition of hemoglobin, products which were not present in cold extracts and which contaminated all of the isolated lipids, it was unknown whether the apparently higher lipid values in the heated extracts were due to further extraction of lipids or only to the presence of the contaminating colored material. There having since been found a satisfactory method of separating this colored matter from the lipids, the present report is concerned with the optimum conditions for extracting lipids from the red blood cells, in general according to the same procedures previously used with plasma and serum.

There have been two common methods for estimating the lipid composition of the red blood cells, the direct and the indirect. In the first the red cells are isolated and an extract made of the weighed or measured cells either with or without saline washing. In the second, the lipid content of whole blood and of plasma is separately estimated and values for the red cells are calculated

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from these in conjunction with the hematocrit reading. Both methods have been extensively used and both have been employed by the author. Values by the indirect method are calculated from at least three experimental figures, each of which is subject to experimental errors which accumulate in the final values for the red blood cells. Hence the direct method is to be preferred, but heretofore it has had the objection that extracts of the red blood cells are invariably deeply colored when prepared in the usual way, this colored material, as already mentioned, having many of the physical properties of lipids.

The red blood cells used in the present investigation were from samples of human blood obtained by mixing together routine specimens received in the laboratories of the Kingston General Hospital. This not only provided a convenient source of material but also eliminated the possibility of individual peculiarities from single samples of blood, and, since values in all experiments tended to be of the same magnitude (*i.e.* about the mean of human red cells), the composite plotting of results was facilitated. The specimens were of oxalated blood and each was allowed to settle and if found unhemolyzed was then used.

The mixed sample was then centrifuged at full speed for $\frac{1}{2}$ to 1 hour or until the red cell layer had become uniformly translucent. This was taken to indicate that the cells had become squarely packed with the elimination of all interstices in which plasma might be present. Repeated washing of such cells with isotonic saline was found to affect their lipid content inappreciably. If differences were encountered between washed and unwashed cells, all the lipids were either increased or decreased to the same relative extent. The lipid composition of plasma is different from that of the red blood cells and inclusion of plasma in a red cell extract would usually increase its neutral fat and cholesterol ester and lower its phospholipid and free cholesterol. When all the lipids of a washed red cell extract were found decreased (or increased) in the same proportion, this must have meant that the saline used in washing was not quite isotonic for the particular sample of cells washed. Hence it was concluded that the saline washing of thoroughly centrifuged red cells is not only unnecessary but may actually introduce an error if the saline is not exactly isotonic with each sample of red blood cells.

A sufficient amount of blood was centrifuged so that, after the plasma was removed, the red cells could be drawn off with a pipette from well below the surface (on which lie the white blood cells of a considerably higher lipid content). Aliquots thus obtained were extracted in various ways but all of the extracts were analyzed by the author's modification of Bloor's oxidative microtechnique as employed in the study of plasma and serum extracts (1). An interesting point in analytical technique arose during the course of this study. A small amount of acetone which had been standing for some time over calcium chloride at the bottom of a large bottle was redistilled and used for the precipitation of phospholipids. The resulting precipitate was of a dirty gray appearance, in contrast to the usual white color, and the calculated phospholipid values were suspected of being low. The acetone was redistilled but this made no difference. The magnesium chloride reagent, which was about 2 years old, was replaced by fresh reagent but this again did not improve the condition. When part of the acetone was allowed to dry again over fresh anhydrous calcium chloride and the results with this compared with the old acetone and with an entirely new dried specimen, it was found that the old acetone had been incompletely dehydrated and that this was responsible for the aberrant low values.

Since the most important factor in the preparation of plasma extracts was found to be the *degree of dilution* in alcohol-ether, this was the first factor studied with the red blood cells. Aliquots of 2 cc. were hemolyzed with an equal volume of distilled water in 125 cc. Erlenmeyer flasks and to this were quickly added amounts of alcohol-ether varying from 5 cc. (a dilution of 2.5 times the original volume of cells) to 120 cc. (a dilution of 60 times). It was found that a finer precipitate of proteins was obtained by quickly adding the solvent to the hemolyzed cells than by adding the hemolyzed cells to the solvent. With dilutions of 10 times or less the proteins tended to clump and had to be broken up with a glass rod, but with greater dilutions a finely divided precipitate was obtained. Unless shaken for a few minutes the protein precipitate even in the greater dilutions tended to settle out and form a solid mass at the bottom of the flask. The extracts were filtered at once without being heated, the precipitate washed once with a small portion of the solvent (so as not to increase solution in the

smaller volumes), and the filtrate and washings made up to volume.

The results of analyses of these extracts for phospholipid, total fatty acid, total cholesterol, and free cholesterol have been shown in Fig. 1 which is a composite presentation of several experiments.

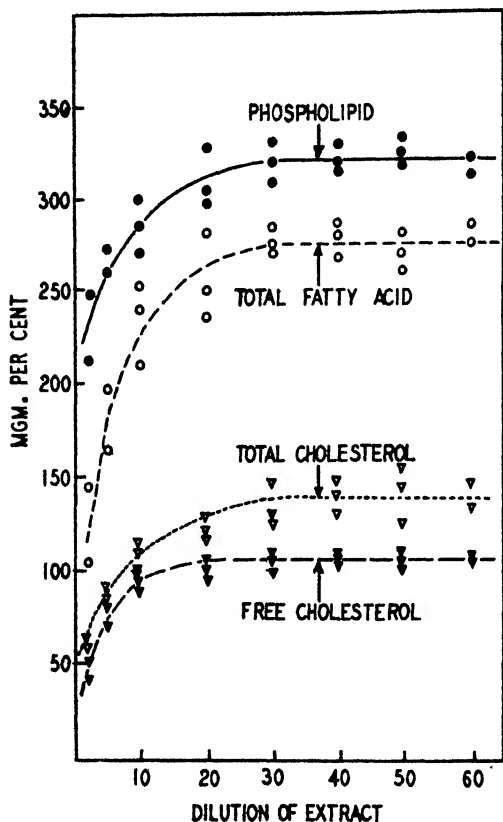


FIG. 1. The relation of lipid composition to the degree of dilution of red blood cells in alcohol-ether.

It may be seen that dilution of the red cells in less than 25 to 30 volumes of alcohol-ether produced incomplete extraction, but that dilutions greater than this did not appreciably affect the recovery. From the data given it may be deduced that neutral fats and cholesterol esters are more difficult to extract with the lesser dilutions than phospholipid and free cholesterol.

The *effect of boiling* the extracts was studied next. 2 cc. of mixed red blood cells were hemolyzed with an equal volume of water and about 60 cc. of alcohol-ether quickly added. Some flasks were left as unheated controls and the remainder were placed on the steam bath and allowed to boil gently for varying periods up to 1 hour. Solvent lost by evaporation was replaced from time to time by fresh alcohol-ether. In preliminary experiments difficulty was encountered with superheating, resulting in boiling over. This was finally overcome by continuously stirring the extracts with a glass rod, especially by dislodging particles of protein which tended to stick to the bottom of the flask. After cooling, the extracts were filtered, washed several times with solvent, the residue pressed out, and the combined filtrates made up to volume. The unheated extracts were colorless but a progressively deeper brownish red developed in the heated extracts. 50 analyses on several mixed samples of human red cells demonstrated that boiling produced apparently higher values for all lipids in the red blood cells, just as was previously found in whole blood (1). The colored matter contaminated all of the isolated lipids and was especially marked in the petroleum ether solution of the total fatty acids, resulting in exceptionally high values for neutral fat.

The problem was how to separate this colored matter from the lipids. By evaporating to dryness portions of the colored alcohol-ether extracts, the colored material was found to be soluble in alcohol, ether, acetone, petroleum ether, methyl alcohol, chloroform, and dilute sodium hydroxide solution; it was insoluble in water and dilute hydrochloric or sulfuric acid. When the colored material was dissolved in 0.1 N NaOH and this extracted with petroleum ether, the color remained in the aqueous alkaline medium and did not pass visibly into the petroleum ether. This was considered as a possible method of separating the colored material from the lipids, but unfortunately the extraction of lipids from the colored alkaline solution was incomplete, especially with regard to phospholipid and free cholesterol. Lipids could be fairly well extracted from an alkaline solution of colorless unheated extracts, so that in some manner the presence of the colored matter hindered the extraction.

While this method was being further investigated, a means was accidentally found whereby the extracts could be freed of colored

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substances. A petroleum ether extract of the saponified and acidified total fatty acids and total cholesterol happened to be left standing overnight and next day the colored material was seen to have completely precipitated out. The petroleum ether solution was filtered, the precipitate repeatedly washed with fresh solvent, and the filtrates were quite clear. Total fatty acid and total cholesterol determined in this extract were found to give values identical with those in unheated control extracts and not higher values, as had always been found in the colored extracts. This experiment was repeated a number of times, some 60 analyses in

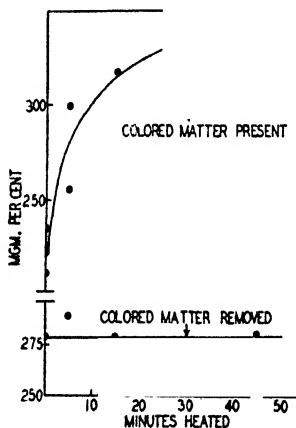


FIG. 2. The total fatty acid content of sufficiently diluted extracts of the red blood cells boiled for varying periods, *before* and *after* removal of the colored material.

all being performed. It was invariably found that when the colored material was thus removed by standing overnight (or longer if necessary) in petroleum ether, the immediately filtered, unheated but sufficiently diluted extracts of the red blood cells contained just as much lipid as the heated ones. A typical experiment illustrating the values for total fatty acid in a colored and a color-free heated extract is shown in Fig. 2. This experiment proved that a sufficient dilution in cold alcohol-ether will extract all lipids from the red blood cells capable of being extracted with this solvent and that heat does not further the extraction of lipids but dissolves colored decomposition products of hemoglobin.

Several *methods of preparation* of the red blood cells for extraction in cold alcohol-ether were next studied. When these cells were added directly to the solvent, the drops from the pipette fell to the bottom in a mass, somewhat resembling the formation of glass beads. Adding alcohol-ether quickly to the untreated cells also produced a clumped precipitate and both of these extracts of untreated red cells gave low lipid values whether or not the massed precipitate was broken up with a glass rod. Hence extraction was carried out on red cells previously (a) hemolyzed with half a volume of distilled water, (b) hemolyzed with 1 volume of distilled water, (c) hemolyzed with 1.5 volumes of distilled water, (d) hemolyzed with 2 volumes of distilled water, and (e) ground with sufficient sand to produce a semidry mass. These several methods of preparation were found to result in almost equally good extraction. No single experiment demonstrated the relative virtue of any one, but from the mean value of a number of experiments (100 analyses) hemolyzing with an equal volume of distilled water was found to give slightly higher lipid values. Hence this latter procedure was adopted in the routine method.

In preparing an alcohol-ether extract of blood lipids, there are two methods in general use for *making up to volume*. One is the distribution method used by Bloor (see previous paper (1)) in which blood is added to the solvent in a volumetric flask and the extract made to volume with the protein precipitate still in the flask. It is believed that the lipids are distributed through the protein precipitate in the same concentration in which they are distributed through the alcohol-ether. In the second method (filtration method), the proteins are filtered off and washed several times with fresh solvent, the combined filtrates being made up to volume. Those who favor the distribution method claim that it is difficult to wash all of the lipid material out of the protein precipitate. An attempt was made to compare these two procedures: Extracts of the red blood cells were prepared as above and made up to volume, one group by the filtration and one by the distribution method. The volume of filtered extract by the distribution method was carefully noted and the total amount of lipid in this determined. To this was added the amount of lipid in the protein precipitate which was calculated to contain the same relative lipid composition as the filtrate. The sum of these two was then

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compared with values obtained by the filtration method, the results being compared in terms of mg. of lipid per 100 cc. of red cells. Curiously enough, the lipid composition of the red cells determined by these two methods was practically the same. To quote figures from one experiment, the values by filtration and by distribution were respectively, total fatty acids 294 and 289 mg. per 100 cc., total cholesterol 144 and 133, free cholesterol 128 and 127, phospholipid 304 and 327. It would appear that both methods are quite satisfactory; in the one case the lipids are equally distributed through the protein precipitate, and in the other case washing

TABLE I

Comparison of Lipid Values in Red Blood Cells from Defibrinated and Oxalated Blood

The results are expressed in mg. per 100 cc. of red blood cells.

Case No.	Blood	Red Blood cells <i>vol. per cent</i>	Total fatty acids	Total choles- terol	Free choles- terol	Phospho- lipid
1	Defibrinated	44.3	268	178	128	351
	Oxalated	40.0	280	190	140	367
2	Defibrinated	49.1	251	144	120	318
	Oxalated	38.2	313	150	130	395
3	Defibrinated	46.0	247	122	116	244
	Oxalated	39.1	306	145	134	377
4	Defibrinated	45.8	267	120	99	270
	Oxalated	43.3	259	132	105	286
5	Defibrinated	47.7	267	122	114	294
	Oxalated	42.8	248	127	115	316

the precipitate thoroughly removes effectively all of the residual lipid material.

Finally, a study was made of the *effect of anticoagulants* on the lipid content of the red blood cells. The lipid composition was determined as above in oxalated and defibrinated samples of the same blood and five such comparisons are given in Table I. In eighteen out of the twenty analyses of directly (not calculated) estimated lipids, the values were higher in the red cells from oxalated blood than from defibrinated blood. In all cases the volume of the red cells was greater in defibrinated than in oxalated blood. Since many of the erythrocytes are invariably caught in

the clot formed during defibrination, this difference in volume was undoubtedly greater than that actually shown. Total fatty acid was the only lipid found higher (in two out of five cases) in the defibrinated cells. Hence it may be concluded that the lipid content of oxalated red blood cells is greater than that of defibrinated cells. This is probably due to absorption of water from the red cells (2), but there were a number of factors here which are being subjected to further study.

SUMMARY

The following method was found to give complete extraction of lipids in the red blood cells in so far as they are soluble in alcohol-ether. Blood is centrifuged until the red cells are translucent when an aliquot is removed from well below the surface. This is hemolyzed with an equal volume of distilled water and 25 to 30 volumes (per 1 volume of red cells) of alcohol-ether quickly added with shaking. Heating is not only unnecessary but undesirable. The extracts are filtered, the residue thoroughly washed, and the filtrate made up to volume. Distribution and filtration methods give practically the same results, but the values are higher in red cells from oxalated than from defibrinated blood.

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THE DETERMINATION OF ETHYL ALCOHOL*

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Of the various chemical methods described in the literature for the determination of ethyl alcohol (1) the majority are based upon the oxidation of the alcohol to acetic acid by means of potassium dichromate in the presence of a high concentration of sulfuric acid. Typical of these methods are the ones devised by Widmark (2) and by Nicloux (3). Widmark reports that it is not possible to calculate the amount of alcohol from the amount of dichromate used, for the oxidation does not lead exclusively to the formation of acetic acid; varying amounts of carbon dioxide and acetaldehyde are simultaneously produced. With solutions of pure alcohol ranging in concentration from 1.43 to 5.16 mg. per cc., he finds that 1 cc. of 0.01 N sodium thiosulfate solution corresponds to 0.1000 to 0.1241 mg. of alcohol. He adopts 0.113, the average of 54 determinations, as the factor to be used in the calculation of the number of mg. of alcohol present in a sample, but it is evident that considerable error is involved in the use of this factor. Furthermore, Widmark's method is limited in its application to samples which contain only relatively small amounts of other volatile substances.

Nicloux attempts to choose conditions such that a maximum yield of acetic acid and a minimum of carbon dioxide and acetaldehyde are produced. He reports a high degree of accuracy under the conditions which he finds optimum, but his method involves the use of three carefully prepared and standardized solutions and

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heating the reaction mixture for 1 hour in a bath thermostatically regulated to maintain a temperature of 85°.

After extensive experience with chromic acid we have turned to the use of potassium permanganate as the oxidizing agent. Chapman and Smith (4) were probably the first to report that, in the presence of alkaline potassium permanganate, ethyl alcohol is oxidized to oxalic acid.¹ Barendrecht (7) determined relatively small quantities of alcohol, 8 to 10 mg., by introducing the sample into a boiling solution of alkaline permanganate. Friedemann and Ritchie (8), in a preliminary study published in 1933, applied a similar principle to the determination of very small quantities of alcohol. Since then, we have studied the method intensively, modified it, and applied it to a variety of biological materials.

In this method the sample is introduced directly into a Kjeldahl flask and distilled from tungstic acid and mercuric sulfate, which precipitate the proteins and prevent foaming. If certain volatile substances other than ethyl alcohol are present, the first distillate is further distilled from alkaline mercuric oxide. The final distillate, or an aliquot thereof, is then oxidized at 100° with potassium permanganate in the presence of sodium hydroxide. After cooling and acidification of the mixture, the excess permanganate is determined iodometrically.

While the oxidation of ethyl alcohol does not proceed quantitatively to carbon dioxide and water under the conditions chosen, yet the yield of other products is low and, what is more important, remains remarkably constant within the limits specified (see Table I). Hence, the use of an empirically determined factor introduces no appreciable error. The final low acidity permits an accurate iodometric determination of the residual oxidizing agent, which is difficult in the case of the iodometric determination of excess dichromate. The end-points are always sharp, even with the 0.005 *N* solutions. A further advantage of the use of potassium permanganate lies in the fact that 1 cc. of 0.01 *N* solution is equivalent to 0.042 mg. of alcohol, whereas with dichromate 1 cc. of 0.01 *N* is equivalent to 0.113 mg. (2). The larger volume of oxidizing agent per given amount of alcohol makes possible greater

¹ For a discussion of the reaction of alcohol with permanganate, see Morris (5). A more complete review of the literature is given by Evans and Day (6).

accuracy in burette readings, and hence, greater sensitivity to small variations in amounts of alcohol present. Moreover, the time required for a complete determination is relatively short, and no special apparatus is required. Finally, the fact that the method can readily be adapted to many types of biological materials permits it a wide range of usefulness.

Description of Method

Apparatus—For the distillation process, a still of small capacity with a minimum of rubber connections is desirable. All of the results reported in this paper were obtained with units constructed as in Fig. 1. The still consists of a Kjeldahl flask,² a glass condenser jacket, and a metal tube. The latter consists of an upper portion of $\frac{3}{8}$ inch tin-coated copper tubing and a lower portion of $\frac{1}{8}$ inch tin-coated brass tubing. A glass collar, for the protection of the receiver from dust and from water which may have collected on the condenser, is made from the upper part of a 6 ounce wide mouth bottle; this is held in place near the end of the delivery tube by means of a loosely fitting rubber stopper. A number of units may be conveniently mounted on a rack of the type described by Friedemann and Graeser (9). Microburners are used as a source of heat.

For work of greater accuracy an all-glass still, with ground glass connections, should be used.

All glassware is carefully cleaned with chromic acid cleaning solution, rinsed well (the final rinsings are with distilled water), and dried on a wire rack in such a way that contamination is avoided. The use of greasy or dusty glassware results in the introduction of large and variable errors.

Reagents—

Distilled water. The water used should be free from organic impurities. It should be stored in a large glass bottle and delivered therefrom through a glass siphon with a minimum of rubber connections. Water from wash bottles should never be used.

Sodium tungstate. A 10 per cent solution.

Mercuric sulfate. 100 gm. of mercuric sulfate are dissolved in

² The rubber stopper should be boiled in dilute alkali and then in distilled water before using.

1 liter of 2 N sulfuric acid. To prevent precipitation of basic mercuric salt, the mercuric sulfate should be added to 500 cc. of water to which have been added 56 cc. of concentrated H_2SO_4 . After heating until solution of the salt is complete, the volume is brought to about 1000 cc.

Calcium hydroxide suspension. 200 gm. of ordinary unslaked lime are slaked with a minimum of water. Then 1 liter of water is added. After a vigorous shaking, the smooth suspension is poured off from the lumps. This is shaken well before using. Lime often

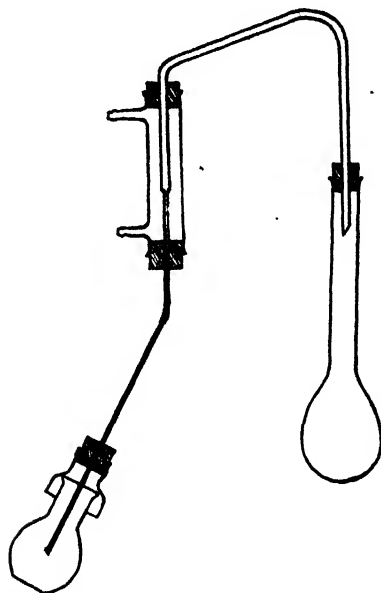


FIG. 1. Single unit of apparatus for determination of alcohol

contains considerable quantities of organic impurities. For more accurate work we recommend the use of $Ca(OH)_2$ prepared from chemically pure CaO . 100 gm. of CaO (reagent quality) are slaked with a minimum of water. To this is then added 1 liter of water. The resulting creamy suspension is kept in a glass-stoppered bottle protected from dust.

5 N sodium hydroxide. Reagent quality should be used. The solution should be stored in a glass-stoppered bottle carefully protected from dust. To prevent resuspension of settled particles

a layer of coarse broken glass, about 1 inch deep, may be introduced.

Potassium permanganate. A solution of 33 gm. per liter is digested 5 or 6 hours on a steam bath and then filtered by suction through asbestos. The first portion of filtrate is discarded. A stock solution, slightly stronger than 0.1 N, is prepared by diluting 100 cc. of the cooled solution to 1 liter. Further dilutions are made when needed.

10 N sulfuric acid.

Potassium iodide. Small crystals; analytical reagent.

Starch solution.

Sodium thiosulfate. The standard 0.1 N solution is diluted to give solutions that are exactly 0.02, 0.01, or 0.005 N.

Procedure

Distillation—The sample is measured (or weighed) into a 300 cc. Kjeldahl flask. To this are added distilled water (sufficient to make the total volume 50 or 60 cc.), 5 cc. of sodium tungstate, 5 cc of mercuric sulfate, and a small amount of powdered talc. The flask is rotated to insure a thorough mixing of the contents, connected to the still, and the mixture brought slowly to the boiling point (there is usually little or no foaming). During the course of 15 to 20 minutes, 30 to 35 cc. are distilled. A 150 cc. fat extraction flask or a 100 cc. volumetric flask may be used as the receiver. In the case of the latter, the volume is adjusted to the mark at the end of the distillation.

With certain samples a second distillation may be necessary in order to remove volatile substances which would interfere in the subsequent oxidation procedure. The initial volume is made about 100 cc.; 60 to 70 cc. of distillate are collected in a Kjeldahl flask containing 5 cc. of mercuric sulfate. A volume of calcium hydroxide, sufficient to impart a deep orange color to the mixture, and powdered talc are added. The flask is rotated (in cases where the aldehyde or ketone content is high, the flask is stoppered and shaken vigorously) and then the distillation is carried out as before.

Oxidation—A 150 cc. fat extraction flask, with a 100 cc. beaker serving as the cover, is used for the oxidation. The sample is introduced either by direct distillation, as described above, or by

pipette from the volumetric flask. In order to determine the size of the aliquot and the strength of the permanganate solution, trial runs may be made. If the aliquot chosen has a volume of 10.0 cc., 15 cc. of water should be added to the extraction flask after the introduction of the sample, for it is important that the total volume during the oxidation should be 60 to 70 cc. (see Table I). To the sample are now added 10 cc. of 5 N sodium hydroxide and 25.0 cc. of a potassium permanganate solution of appropriate strength. More consistent results are obtained if the permanganate is added with constant rotation. In ordinary work, where moderately large quantities of alcohol are expected, a 0.02 N solution should be used. After further rotation to insure thorough mixing of the contents, the flask is placed in a boiling water bath. At the end of 20 minutes, it is removed to a bath of cold running water. As soon as it is cool, 10 cc. of 10 N sulfuric acid are added. After a few more minutes of cooling, the flask is removed; 0.2 to 0.5 gm. of potassium iodide is added, and the iodine titrated with standard sodium thiosulfate of approximately the same strength as the permanganate. 1 to 2 cc. of starch solution are added when the color of the solution is a pale yellow. The titration is continued until the solution is colorless.

Blank determinations should be made simultaneously.

Calculations— A (cc. of thiosulfate required for the blank) minus B (cc. of thiosulfate required for the sample) equals C (cc. of thiosulfate equivalent to the potassium permanganate used in the oxidation).

The amount of alcohol present in the solution oxidized can now be calculated. 1.00 cc. of 0.020 N KMnO_4 = 0.0855 mg. of ethyl alcohol, or 0.00186 mm; 1.00 cc. of 0.010 N KMnO_4 = 0.0420 mg., or 0.000912 mm; 1.0 cc. of 0.005 N KMnO_4 = 0.0215 mg. or 0.000467 mm. These factors apply only when C is less than 5 cc. (6 cc. in the case of the 0.02 N KMnO_4). If C is greater than 5 cc., approximate results may be obtained by using factors given in Table I.

If S is the number of cc. of initial sample equivalent to the aliquot taken for oxidation, the following formulæ give the amount of alcohol in mg. per cent.

$$\begin{aligned} C \times 8.55/S &= \text{mg. \% alcohol, with 0.020 N } \text{KMnO}_4 \\ " \times 4.20/" &= " \% " " 0.010 " " \\ " \times 2.15/" &= " \% " " 0.005 " " \end{aligned}$$

TABLE I

Oxidation of Pure Ethyl Alcohol

25.0 or 35.0 cc. of alcohol solution, 10 cc. of 5N NaOH, 25.0 cc. of KMnO_4 ; total volume, 60 or 70 cc. Heated 20 minutes in a boiling water bath. Water blanks = 25.0 to 25.6 cc. of 0.020, 0.010, or 0.005 N thiosulfate.

Alcohol oxidised	0.02 N KMnO_4			0.01 N KMnO_4			0.005 N KMnO_4		
	Total vol- ume 60 cc.	Total vol- ume 70 cc.	Alcohol equiva- lent	Total vol- ume 60 cc.	Total vol- ume 70 cc.	Alcohol equiva- lent	Total vol- ume 60 cc.	Total vol- ume 70 cc.	Alcohol equiva- lent
mg.	cc. KMnO_4	cc. KMnO_4	mg. per cc. "	cc. KMnO_4	cc. KMnO_4	mg. per cc.	cc. KMnO_4	cc. KMnO_4	mg. per cc.
0.796	8.53	8.32	0.0933						
0.778	7.89	7.76	0.0986						
0.716	7.96	7.65	0.0900						
0.700	7.41	7.35	0.0945						
0.637	7.30	7.09	0.0873						
0.622	6.84	6.72	0.0909						
0.557	6.56		0.0849						
0.544	6.21		0.0876						
0.517	6.17	6.14	0.0838						
0.466	5.46	5.50	0.0853						
0.438	5.12		0.0855						
0.398	4.71		0.0845						
0.389	4.55		0.0855						
0.350				6.64		0.0527			
0.348				6.82		0.0510			
0.311	3.62		0.0859	6.51	6.10	0.0478			
0.305				6.32		0.0483			
0.272				5.88		0.0463			
0.233	2.74		0.0850	5.36	5.24	0.0435			
0.218				5.08	5.07	0.0429			
0.194				4.68		0.0415	6.13	5.43	0.0316
0.174				4.15	4.18	0.0419			
0.155	1.82		0.0852	3.68	3.68	0.0421	5.66	5.10	0.0274
0.131				3.12		0.0420			
0.117							5.08	4.53	0.0230
0.087							3.88	3.65	0.0224
0.078	0.89		0.0876*	1.83		0.0426	3.42	3.30	0.0228
0.0700				1.65		0.0424			
0.0697							3.25	3.09	0.0215
0.0623				1.51		0.0413	2.90	2.63	0.0215
0.0610							2.91	2.69	0.0210
0.0545				1.31		0.0416			
0.0467				1.15		0.0406	2.24	2.07	0.0208
0.0389				0.93		0.0418*			
0.0311							1.50	1.30	0.0207
0.0156							0.85	0.63	0.0184*

* Not included in any calculations.

Precautions—Inasmuch as the success of the method depends upon the controlled oxidation of a minute quantity of an organic compound (ethyl alcohol), it is absolutely essential that all other oxidizable substances be excluded. In practically every instance those who have described oxidimetric methods have stressed the importance of clean glassware. We too urge that great care be taken to use glassware that is free from dust and grease. Cleaning with chromic-sulfuric acid mixture (with not less than 80 per cent H_2SO_4) is recommended. It is also important that the distilled water be of good quality. If organic impurities are present in the water, it is impossible to obtain accurate results.

The rubber stopper used to connect the Kjeldahl flask with the condenser may be the source of considerable volatile oxidizable material. The amount is small and fairly constant, however, (so that a correction for this error is provided by the blank) if the still is steamed out each day before a series of distillations is begun.

The reagents for the oxidation should be measured with pipettes, and not burettes. It is well to plug all pipettes with cotton in order to avoid contamination of the solutions with chance droplets of saliva. The lower part of these pipettes should not be allowed to touch the desk, nor should the lower part of these pipettes be touched with the hands, and, if used repeatedly, each pipette should be placed in an upright position in a test-tube.

Saliva—If a beverage or solution containing alcohol has been taken orally, the mouth should be rinsed well with several changes of water in order to remove the last traces of the liquid. At least 5 minutes should then elapse before a sample of saliva is collected for analysis. A rapid flow of saliva may be induced by chewing a small piece of paraffin. Chewing gum should never be used. The sample should be preserved by the addition of sodium fluoride (0.1 gm. for 10 cc. of saliva). 1 cc. samples are then distilled according to the directions given above. A second distillation is unnecessary (see Table V).

Blood—In the collection of samples, the skin is cleansed with soap and with 0.1 per cent mercuric chloride solution. If 0.2 cc. samples are desired, the skin is pricked as in making a blood count. 10 drops or more are allowed to run into a small test-tube containing a small amount (0.1 to 0.2 per cent) of a mixture of equal parts of powdered sodium fluoride and potassium oxalate. For the

collection of larger samples, a dry sterile syringe for venous puncture is used. After collection of the blood, the container is tightly stoppered and immediately placed in the refrigerator. The analysis should be undertaken as soon as possible, at least within 24 hours after collection.

In the measurement of 0.2 cc. samples, a pipette of 0.2 cc. capacity, such as is used in the measurement of the Kahn antigen, has been found to give fairly accurate results (see Table III). Blood is drawn up past the mark; the tip is wiped with a towel, and the meniscus adjusted to the mark. The pipette is then allowed to empty slowly into the Kjeldahl flask (time necessary, about 1 minute); the last drop is blown out. The distillation from the sodium tungstate and mercuric sulfate is then carried out, with a 150 cc. extraction flask as the receiver.

Whenever larger amounts of blood are available, 1 cc. samples are taken. The pipettes should be allowed to drain slowly. The 100 cc. volumetric flask is used as the receiver, and a suitable aliquot of the distillate taken for oxidation. In the case of blood, as with saliva, a redistillation is not required, because of the presence of only very small amounts of interfering substances.

Urine—The samples are preserved with sodium fluoride (1 gm. per 100 cc.). Since urine often contains rather large amounts of volatile reducing materials, especially in the case of diabetic individuals, the two distillations outlined above are necessary in order to obtain accurate values for alcohol content. 1 cc. samples are generally used, although 5 or 10 cc. may be taken if the amount of alcohol is low.

Culture Media—Sulfuric acid is added to stop the metabolic activities of microorganisms as recommended by Friedemann and Brook (10). 5 or 10 cc. samples are taken for analysis; two distillations are required because of the frequent occurrence of aldehydes or ketones as products of metabolism. Samples of the same culture medium in which no microorganisms have grown are run in order to obtain blank values. Certain bacteria produce other alcohols besides ethyl alcohol. Our method is fairly specific for alcohols as a group, but it cannot be used to determine ethyl alcohol when other alcohols are also present.

Tissues—The sample is frozen in liquid air and crushed according to the procedure of Graesser, Ginsberg, and Friedemann (11).

A weighed sample of the crushed tissue is introduced directly into the Kjeldahl flask and treated like a sample of blood.

EXPERIMENTAL

Oxidation of Pure Ethyl Alcohol—Commercial absolute ethyl alcohol was heated with unslaked lime under a reflux condenser for 15 hours. The alcohol was then distilled; only the middle third of the distillate was retained. Solutions of known composition by weight (approximately 15 per cent) were prepared, and these were immediately diluted to form 1 per cent solutions. The latter were kept in the refrigerator and diluted further as needed. The oxidations were carried out as described above, with 25 cc. of the alcohol solution, 10 cc. of 5 N sodium hydroxide, and 25 cc. of potassium permanganate. Oxidations were also carried out

TABLE II
Recovery of Ethyl Alcohol after Distillation

Weight of alcohol distilled	Distillation from $\text{HgSO}_4 + \text{Na}_2\text{WO}_4$ (1)	Distillation from $\text{HgSO}_4 + \text{Ca}(\text{OH})_2$ (2)	Two distillations; (1) followed by (2)
mg.	per cent recovery	per cent recovery	per cent recovery
1.560	98	100	99
0.936	101	103	99
0.780	93	98	99
0.312	97	96	100

in which the sample (25.0 cc.) was further diluted with 10 cc. of water before the addition of the alkali and permanganate. The study covered a wide range of concentrations of alcohol and three concentrations of potassium permanganate. Typical results are shown in Table I.

With a given permanganate solution, the results are fairly constant until the volume of oxidizing agent used exceeds 5 cc. (6 cc. in the case of the 0.02 N KMnO_4). Thus, in the case of the data presented in Table I, the greatest deviations from the average are -1.2 and $+0.5$ per cent with 0.02 N KMnO_4 , and -3.3 and $+2.1$ per cent with 0.01 N KMnO_4 . It may be noted that the values change rapidly when the volume used in the oxidation is greater than 5 (or 6) cc. In no case is the alcohol oxidized completely to carbon dioxide and water; the calculated equivalents are 0.0768,

0.0384, and 0.0192 mg., for 0.02, 0.01, and 0.005 N solutions, respectively.

We have chosen as the alkali concentration one which permits the oxidation to proceed most nearly to completion. With higher or lower concentrations, the extent of the oxidation rapidly diminishes.

Distillation. Effect of Various Agents on the Recovery—Alcohol solutions of varying concentrations were distilled according to the procedure outlined above. Typical results are given in Table II. It is evident that the reagents used remove little if any of the alcohol. The average recovery in all determinations shown in Table II is 98.6 per cent. Low recoveries, such as 93 per cent in the single distillation in the second column, are rarely encountered if considerably more than one-half of the solution is distilled.

Recovery of Alcohol from Blood and Urine—1 cc. samples of blood were measured into Kjeldahl flasks. To these were added 1 cc. samples of alcohol solutions varying in concentration from 79 to 278 mg. per cent. Distillations were carried out from the mercuric sulfate and sodium tungstate reagents, and the appropriate aliquots of the distillates oxidized. 0.2 cc. samples of blood and alcohol solutions were treated similarly, except that the distillates were collected in extraction flasks. The results are shown in Table III. In the case of the smaller samples, it will be noted that the recovery was somewhat high and variable. Because the error in the measurement of small quantities of liquid may be rather large, the pipettes should be carefully calibrated or the samples weighed whenever a higher degree of accuracy is desired.

To 1 cc. samples of normal urine amounts of alcohol varying from 63 to 156 mg. per cent were added. The recovery here was excellent, just as in the case of blood.

Removal of Interfering Substances—Various methods have been recommended by different workers for the removal of aldehydes and ketones, two classes of substances which are very similar to alcohol in volatility and ease of oxidation. Gorr and Wagner (12) review the literature up to 1925 and then outline a new procedure. The sample is heated under a reflux condenser with a mixture of mercuric chloride and slightly less than the equivalent amount of alkali. Pelgroms (13) merely distills slowly from mercuric oxide. He does not describe his method in detail or state the alkali con-

centration used. We have made a careful study of the use of mercuric oxide and its efficiency in the removal of aldehydes and ketones, and we find that it is a very satisfactory reagent for the purpose. We recommend the use of excess of calcium hydroxide. The alcohol is recovered practically quantitatively (Table IV) at

TABLE III
Recovery of Added Alcohol from Blood and Urine

	Sample	Alcohol added	Recovery		Sample	Alcohol added	Recovery
	cc.	mg. per cent	per cent		cc.	mg. per cent	per cent
Blood	1.00	79	101	Urine A	1.00	63	96
	1.00	119	99		1.00	78	97
	1.00	159	101		1.00	91	102
	1.00	198	100		1.00	156	100
	1.00	278	103	Urine B	1.00	63	98
	0.20	79	106		1.00	78	99
	0.20	119	103		1.00	91	100
	0.20	159	101		1.00	156	99
	0.20	198	107				

TABLE IV
Removal of Acetaldehyde and Acetone

The procedure outlined for urine was followed.

Ethyl alcohol used	Substance added	Ethyl alcohol recovered
mg.	mg.	mg. per cent
1.49	Acetaldehyde 1.1	102
0.87	“ 1.1	102
0.75	“ 1.1	105
1.49	Acetone 1.0	99
0.87	“ 1.0	99
0.75	“ 1.0	100
0.29	“ 1.0	100

this alkalinity when mixed with equal or larger quantities of acetone or acetaldehyde. At least 99 per cent of the acetone is removed, and acetaldehyde is removed to the extent of 95 to 98 per cent under the conditions specified.

Whenever complex mixtures are dealt with, the first distillation from an acid medium results in the removal of volatile basic

substances, such as the amines, and the second, from the alkaline mercuric oxide, permits the removal of phenols and the lower aliphatic acids, aldehydes, and ketones. The method therefore provides an excellent separation of alcohol from other substances commonly present in biological materials.

Blank Values of Blood, Saliva, and Urine—The quantity of oxidizable material obtained by the distillation, according to our procedure, of 1 cc. of normal blood, saliva, or urine is very small. Thus, in the case of urine, the volume of 0.01 N potassium permanganate used in the oxidation of a 25 cc. aliquot of the distillate varies from 0.02 to 0.10 cc. The same values are obtained with diabetic urines. In the case of a diabetic urine, four determinations with 1 cc. samples gave an average titration of 0.05 cc., equivalent to about 0.8 mg. per cent of ethyl alcohol, whereas the same urine, when distilled without any reagents whatsoever, gave a titration of 3.30 cc., equivalent to 55 mg. per cent of alcohol. With saliva and blood, just as with urine, the blank values are very small. Hence, in ordinary work it is unnecessary to apply corrections for the oxidizable material present in the samples.

Normal Alcohol Content of Blood, Saliva, and Urine—That ethyl alcohol is normally present in blood and tissues has been demonstrated conclusively by Gettler, Niederl, and Benedetti-Pichler (14). These workers isolated the alcohol, identified it by means of its physical properties, and prepared characteristic derivatives. Using their quantitative microethoxy method, which is specific for alcohols, they have obtained the following average values for the normal alcohol content of blood: human, 4 mg. per cent; dog, 1.3 mg. per cent.³ Their results are the lowest thus far published.

Our method gives the results summarized in the last column of Table V. It is to be noted that there is a fairly close agreement among the values obtained for the three body fluids, blood, urine, and saliva, despite the fact that the content of volatile materials varies widely, being greatest in urine.

The data in the third column of Table V have been included in order to show the necessity for two distillations in the case of urine. Single distillations gave high and variable results. It may also be

³ A summary of determinations by previous workers of the normal alcohol content of tissues and body fluids is given by Gettler, Niederl, and Benedetti-Pichler.

noted that in certain instances the quantities of reagents used were not sufficient for the volume of urine taken. Thus, the 20 cc. sample of diabetic urine, after two distillations, yielded 1.38 mg. per cent of oxidizable material calculated as ethyl alcohol, whereas approximately one-half as much was obtained from the 10 and 5 cc. samples. Again, normal Urines U and V yielded 8.9 and 6.0

TABLE V

Normal Alcohol Content of Urine, Blood, and Saliva

With the exception of blood (see below) the quantity of reagents used in these analyses was that recommended for 1 cc. of sample.

Description of sample	Volume of sample	Treatment	
		Distillation from $\text{HgSO}_4 + \text{Na}_2\text{WO}_4$	(1) followed by distillation from $\text{HgSO}_4 + \text{Ca}(\text{OH})_2$
		(1)	(2)
	cc.	mg. per cent	mg. per cent
Urine A, normal	20	2.0	0.77
“ B, “	20	4.4	0.50
“ R, diabetic*	20		1.38
	10		0.53
	5		0.74
	1	17.3	0.8
“ U, normal	10	8.9	0.92
“ V, “	10	6.0	0.62
Blood A, normal†	10		0.10
	10		0.15
“ B, “ †	10		0.17
	10		0.40
	10		0.40
Saliva A, normal	10	0.38	0.32
“ B, “	10	0.36	0.31

* This sample contained much glucose and gave a strong reaction with ferric chloride (acetoacetic acid).

† The first distillation was carried out with 10 cc. of acid HgSO_4 and 40 cc. of Na_2WO_4 in a total volume of 100 cc.

mg. per cent, respectively, after the first distillation; after two distillations, they gave 0.92 and 0.62 mg. per cent. With an excess of mercuric sulfate (5 gm. of HgSO_4 added to the mercury-tungstate mixture), practically the same results (not shown in Table V) were obtained after one distillation as with the two; *viz.*, 0.81 and 0.96 mg. per cent.

SUMMARY

A method for the determination of ethyl alcohol in biological materials has been outlined. The alcohol is separated from other constituents by distillation after addition of reagents which remove many of the commonly occurring volatile substances. The distillate, or an aliquot thereof, is oxidized in a water bath with alkaline permanganate for a period of 20 minutes. After the mixture has been cooled, it is acidified, and the excess permanganate determined iodometrically. The necessity for cleanliness of glassware and purity of water has been emphasized. The high degree of reliability of the method is indicated by the following criteria: (a) practically quantitative recovery of alcohol from pure solutions, (b) practically quantitative recovery of alcohol when added to blood and urine, (c) excellent removal of many of the common volatile substances which occur in biological materials, and finally (d) obtaining a low and consistent value, approximately 0.4 mg. per cent, for the normal alcohol content of blood, saliva, and urine.

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A STABILIZED PHOTOELECTRIC COLORIMETER WITH LIGHT FILTERS*

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Photoelectric colorimeters for biological work fall into two main classes. The first is the single photocell, direct reading photoelectric photometer, of which the Sheard and Sanford photometer (6-8) may be taken as an example. The second is the double photocell, null point type, such as the comparison photometer of Goudsmit and Summerson (2). Quite apart from obvious advantages of simplicity of design and operation, the direct reading type is to be preferred, since it allows full use to be made of the advantages inherent in the application of color filters to the colorimetry of complex media. This type of instrument can, however, only be successful when it employs a source of light of absolutely constant intensity. This requirement introduces a technical difficulty which has been the cause of failure of most single cell colorimeters, and an attempt to evade the difficulty has led to the adoption of the double photocell principle. However, this does not by any means solve the problem of stability, since the resulting instrument exhibits erratic behavior as a result of unavoidable asymmetry of the response of the two cells to light of different wavelengths.

These, then, are the primary sources of error peculiar to the two types of photoelectric colorimeter, but superimposed on them is another fault shared equally by instruments of both classes, namely

* This instrument was demonstrated at the annual meeting of the Federation of American Societies for Experimental Biology, Washington, D. C., March 25-28, 1936.

† The work has been aided by grants from The Banting Research Foundation, and from the Loomis Laboratories, Tuxedo Park, New York.

inconvenience of operation due to purely mechanical causes. The instrument to be described in this paper is a single photocell colorimeter in which extreme stability of light intensity has been achieved by a simple unconventional design. This design has not only eliminated errors due to light fluctuation, but has made possible a degree of simplicity and convenience of operation not hitherto achieved in any colorimeter, either visual or photoelectric. Moreover, the use of a truly constant source of illumination has made it possible to enlarge the field of application of light filters to cover most of the problems formerly requiring the use of the spectrophotometer, as well as other procedures for which not even the spectrophotometer is available. These new procedures will be described in a series of papers of which the present one deals with the design and operation of the apparatus, and indicates the nature and extent of its use in biological colorimetry.

General Considerations

The general principles of the single cell photoelectric colorimeter are briefly as follows: A beam of light (whose intensity can be varied at will, but which can be kept constant at any desired value) falls on a photocell which produces a deflection in a galvanometer to which it is connected. If an absorption cell containing a colored solution is placed between lamp and photocell, the percentage of light transmitted through the solution is proportional to the ratio of the final to the initial galvanometer deflection. The concentration of the colored substance in the sample can then be read from a chart showing the variation of light transmission with concentration. No assumption need be made as to the nature of the relation between light intensity and galvanometer deflection, nor between deflection and concentration. As long as the apparatus is constant in behavior, a colored solution which corresponds to a particular galvanometer reading at the time of calibration will give the same reading at any future time. Increased accuracy may be obtained by using color filters which transmit only that portion of the spectrum in which the solution has the correct degree of absorption (Kennedy (3), Exton (1), Sheard and Sanford (8), Koller (4), Millikan (5)). Indeed, the color filter technique can be refined to such an extent that it not only improves the accuracy of existing procedures, but renders entirely new ones possible.

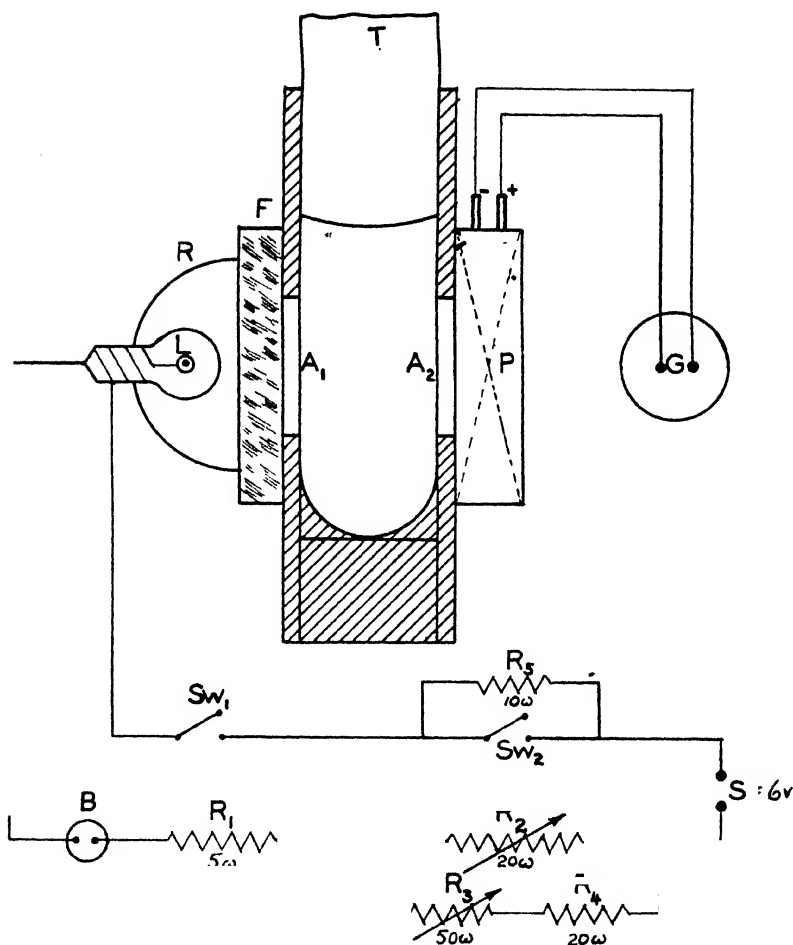


FIG. 1. Diagrammatic cross-section showing electrical circuit. A_1 , A_2 , rectangular apertures 1 inch high by $\frac{1}{16}$ inch wide; B , insulated binding post; F , glass color filter (Corning or Jena glass); G , galvanometer; L , Mazda No. 31 flash-light bulb; P , General Electric blocking layer photocell; R , 1.5 inch diameter aluminum reflector; R_1 to R_5 , control resistances; S , 6 volt storage battery, dry battery; or voltage-regulating transformer; Sw_1 , main lamp switch; Sw_2 , low range lamp switch; T , $7 \times \frac{1}{4}$ inch soft glass test-tube.

Description of Apparatus

In Fig. 1 is shown a schematic cross-section through the body of the colorimeter, together with a diagram of the simple electrical circuit.

A beam of light from the lamp L in the reflector R passes through the color filter F , and then through the colored solution in the absorption tube T , which is mounted in a suitable holder between the rectangular apertures A_1 and A_2 which define the cross-section of the light beam. The transmitted beam falls on the photocell P , the current from which is indicated on the galvanometer G . The lamp is energized by the power supply S , and the intensity of the beam is controlled by the rheostats R_2 and R_3 . The legend to Fig. 1 contains the essential facts about the more important components. One must, however, emphasize the fact that successful operation of the apparatus depends on rigid adherence to a definite set of optimum characteristics, not only in the electrical components but also in the mechanical assembly. Although such details cannot be given here,¹ a few additional notes on the various components are required to make clear the more important features and advantages of the design.

Source of Light—This consists of a Mazda No. 31 flash-light bulb mounted in a hemispherical matte-surfaced aluminum reflector. The bulbs are individually selected for uniform physical and electrical characteristics, so as to be interchangeable without affecting the calibration of the instrument. The bulbs are run so far below their rated voltage that their useful life is equivalent to almost a year of hard service.

This simple arrangement has many important advantages. The power requirement (1 watt) and the current drain (200 milliamperes) are so low that the lamp can be energized by a 6 volt storage battery. This insures extreme stability over long periods of time. The small current in the lamp circuit also simplifies the problem of controlling the light intensity, since heating effects in rheostats are at a minimum. The light intensity can be varied over an extremely wide range (200-fold), thus allowing the use of filters of widely varying densities.

Power Supply—For best results and maximum stability a 6 volt

¹ The author will be pleased to supply full constructional details and drawings to anyone who cares to write to ask for them.

lead storage battery is used. If this high degree of stability is not required, a standard 6 volt voltage-regulating transformer may be substituted, especially in localities where well controlled alternating current is available. Where portability is essential, satisfactory performance can be obtained with a 6 volt dry battery.

The light intensity can be varied smoothly over a wide range by the simple control circuit shown in Fig. 1. The only manual controls are the two switches and the rheostats R_2 and R_3 which provide coarse and fine adjustment respectively. The fixed resistor, R_1 , limits the maximum voltage on the lamp to 5 volts, thus allowing an ample safety factor over the normal operating voltage of 6.2 volts. The switches Sw_1 and Sw_2 are of the low contact resistance, mercury-to-platinum type.

Color Filters—The glass color filters are mounted in brass filter holders (two filters to each holder) which can be moved up and down in a slot between the lamp and the absorption tube. Additional filters are kept on hand in extra interchangeable filter holders. Suitable filters for every problem can be prepared by using various combinations of different thicknesses of Corning and Jena glasses. These glasses are more stable than gelatin films, and are accurately reproducible by spectrophotometric standardization. The theoretical basis of the use of light filters in colorimetry, the mathematical theory of the photoelectric colorimeter with light filters (by analogy with spectrophotometer theory), and the technique of selecting filters for various problems will be fully discussed in later papers of this series. By careful selection it is possible to acquire a small set of filters which will cover a wide range of colorimetric procedures. In order to be able to select a combination of filters to isolate any desired region in the spectrum (down to bands only $40\text{ m}\mu$ wide), it is necessary to accumulate a large stock of various thicknesses of all the sharp cut-off filters available. As long as one such set of filters exists, it is unnecessary for the individual user of the colorimeter to make a collection of his own, since when once the correct filter has been chosen, it can be duplicated as often as necessary from the original data.

Absorption Cell—Instead of the conventional rectangular cemented glass cells, standard $7 \times \frac{7}{8}$ inch, round bottomed, soft glass test-tubes are used. The absorption tube fits into a bakelite sleeve which lines a brass tube, to the flattened sides of which the

other parts of the instrument are soldered. Interchangeable bakelite tubes with diaphragms of different sizes allow the use of samples of 6, 8, or 10 cc. instead of the usual 14 cc. Tubes of uniform dimensions are obtained from the makers, and final selection is made by filling all the tubes with the same colored solution, reading in the colorimeter, and discarding those which vary by more than 0.5 per cent from the mean. In this way one may easily obtain a set of 100 or more matched tubes which are inexpensive, convenient to handle, and easy to keep clean.

The use of standard test-tubes greatly simplifies the operation and construction of the colorimeter. Since many interchangeable tubes are available, one may carry out the entire preliminary chemical procedure (except in a few special cases) in the same tube in which the final colorimetric reading is to be made. As the act of making a reading does not in any way interfere with the solution under test, serial readings on large numbers of samples may be made as often as desired. This is particularly important in the case of volatile media. The ability to make rapid serial readings on numerous samples is invaluable in the study of the effect on color reactions of time, temperature, pH, and other variables.

Photoelectric Cell—The General Electric blocking layer photocell has been found most suitable. The cells must be selected to have approximately equal output under conditions existing in the colorimeter, but this is a convenience rather than a necessity, since each instrument is individually calibrated. By eliminating any possibility of heating effects, and by exposing the photocell to very low intensities of illumination (less than 1 foot candle), photoelectric fatigue and temperature effects have been rendered negligible. In this connection it should be pointed out that a great deal of the instability of certain photoelectric devices, commonly attributed to "photoelectric fatigue," is really due to heating effects in overloaded lamp circuits.

Galvanometer—The galvanometer should have a period of 3 seconds or less, a coil resistance of about 1000 ohms, and an external critical damping resistance of about 5000 ohms. A full scale deflection of 100 divisions should correspond to a current of about 2.5 microamperes. When many readings must be made at each sitting, a minimum of fatigue for the operator is assured by using an enclosed lamp and scale type of galvanometer, mounted on a

rigid shelf at the level of the seated observer's eye. The Rubicon type 3403 D.C. spot light galvanometer is most satisfactory. For a portable model a standard 15 microampere needle type meter (Weston, No. 440) is convenient. The photoelectric cell is connected directly to the galvanometer, for which it supplies a suitable

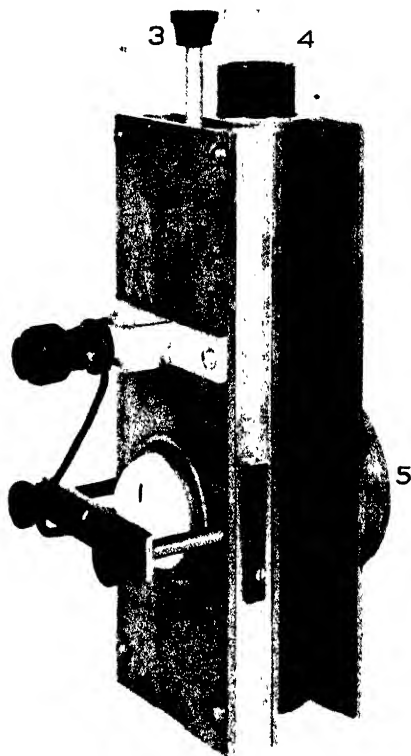


FIG. 2. Essential structural unit of the colorimeter. 1, lamp and reflector; 2, filter compartment; 3, handle of filter slide; 4, test-tube in holder; 5, photocell.

external damping resistance. Since the calibration will be slightly influenced by the galvanometer coil resistance, interchangeable results will only be obtained when everyone uses the same type of galvanometer. This is of course not essential, as long as each apparatus is individually calibrated.

Assembly of the Apparatus—The main structural unit shown in Fig. 2 has no moving parts except the filter slide. A simple method of mounting the lamp and reflector makes it possible to change the lamp in a few seconds. The device may easily be duplicated in any workshop, and so long as the main unit is built rigidly to the proper specifications, the final assembly is entirely a matter of choice. All the components may conveniently be

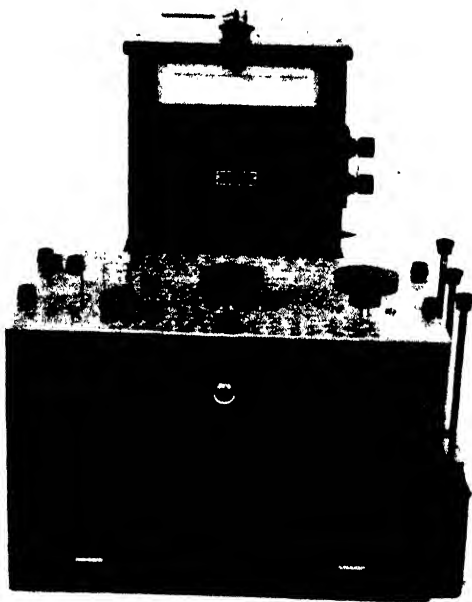


FIG. 3. Photograph of complete colorimeter. The tube holder and filter slide can be seen piercing the panel. The switches, control knobs, and spare filter holders can also be recognized.

mounted on an aluminum panel which forms the cover of a wooden box; a door in the front of the box gives access to the interior when the lamp has to be changed (see Fig. 3).

Operation of the Apparatus

Before summarizing the very simple routine employed in using the colorimeter, it is necessary to call attention to one characteristic feature which is due to the use of a test-tube as the absorption

cell. When a blank tube filled with a colorless solvent such as water is placed in the holder, it acts as a cylindrical lens which concentrates the light beam on the photocell. The galvanometer reading with a blank tube (referred to as the "blank setting") is, therefore, greater than the corresponding reading with the holder empty (referred to as the "center setting"). The ratio between the two is about 1.5, and is determined by the geometry of the system, the wave-length of the light, and the refractive index of the contents of the tube. Since this ratio is constant for any one type of determination, it is immaterial whether the initial deflection is adjusted with the holder empty, or with a blank tube (containing solvent only) in place.

The operation of the colorimeter may now be summarized. The proper filter is selected, a blank tube containing pure solvent is inserted, and the rheostats are adjusted until an initial deflection of 100 divisions is obtained. The blank tube is now replaced by the sample tube, the new deflection is noted, and the corresponding value of the concentration is obtained from the proper calibration chart. After each reading the galvanometer returns to the original center setting, and the operator soon develops the habit of almost automatically checking its stability before inserting a new tube. This safeguard is valuable, although even this slight automatic readjustment of the setting should not be necessary in a series of twenty readings. The speed with which readings can be made is limited only by the time taken by the galvanometer to make the necessary excursion, so that ten readings per minute can be made with ease.

In calibrating the apparatus one makes up carefully and in triplicate a series of standards of different strengths to cover the desired range, and plots the best curve possible through the resulting points. No attempt should be made to read the galvanometer more closely than to the nearest half division, since this furnishes as much accuracy as can be expected from most colorimetric procedures. Since no color standards are required from day to day, the net result of this method of calibration is that the accuracy of each day's results is maintained at a level corresponding to the greatest accuracy attainable under the most favorable conditions. In future papers dealing with various procedures which have already been investigated, full instructions will be given as

to the method of preparing a set of standards for calibration which will give an evenly spaced array of points.

Application of the Apparatus

A single pair of filters has been chosen with just the right degree of sensitivity to allow the apparatus to be used in place of the Duboscq colorimeter in all the standard colorimetric procedures, without the introduction of any modifications except the elimination of color standards.

When the most highly selective filters possible are used, the perfect stability of the readings allows accurate determination in extremely pale solutions. This has made possible a series of micromethods in which the useful range of various standard procedures has been extended far below the lower limits of visual colorimetry. The application of the apparatus to the accurate detection of the end-points of titrations is self-evident. The infra-red sensitivity of the photocell has encouraged the investigation of the "colorimetry" of nearly colorless solutions which have strong absorption in the infra-red but little or none in the visible region.

Studies of the behavior and velocity of color reactions are facilitated by the direct reading nature of the device, and by one's ability to make serial readings on large numbers of tubes in such a way that the act of making a reading does not in any way disturb the solution or necessitate the making of an adjustment to the apparatus before the next reading can be made. Rapid serial readings are invaluable in the quantitative determination of rapidly changing colors, since the point of maximum color development can be accurately determined.

By taking advantage of the wide range and reserve capacity of the illuminating system, highly selective filters can be used when it is necessary to eliminate the interfering effects of extraneous colors, or to determine independently both components of a mixture. It is in this field of heterochromatic colorimetry (for example, the analysis of mixtures of hemoglobin derivatives) that the instrument has found its widest application. Apart from its use for quantitative colorimetry, the apparatus may be used as a qualitative colorimeter for color matching and specification, by means of approximate trichromatic coordinates based on measure-

ments in three narrow spectral regions corresponding to the three primary colors.

Being essentially a photoelectric photometer, the apparatus may also be used as a nephelometer, especially in colored media in which the error due to absorption by the color itself can be eliminated by the use of suitable filters. For example, nephelometric measurements on red blood cell suspensions (made with a red filter to eliminate hemoglobin absorption) have been applied to the making of approximate rapid red cell counts, to the study of the kinetics of hemolysis, and to the determination of the fragility of red blood cells.

Results

The essential factors in the performance of the colorimeter on which data must be given are (1) precision (reproducibility of readings on duplicate samples), (2) accuracy (actual deviation from the true result), and (3) permanence of calibration (reproducibility of readings on the same sample from day to day). Data on all these points can best be given by reference to a single specific procedure. The determination of hemoglobin has been chosen because it involves no complex chemical manipulations, so that any errors which occur must be due entirely to defects in the instrument itself. Moreover, the Van Slyke oxygen capacity method provides a standard of known accuracy for purposes of comparison.

From the data presented in Table I, the following conclusions may be drawn with regard to the three factors listed above.

Precision—The figures in the second column of Table I indicate that the range of variation in samples set up in duplicate never exceeds 1 per cent. In terms of actual galvanometer readings this corresponds to one-half of 1 scale division. This may be taken as the maximum allowable variation, and is due almost entirely to practical limitations in the accuracy with which sets of matched tubes may be selected. None of this variation is due to random fluctuation in the light source, as this would never produce an alteration in the blank setting of as much as 0.05 division (in 100) during the time of making a reading.

Accuracy—The figures in the third column of Table I indicate that the probable error between the average of duplicate Van

Slyke and photoelectric determinations is of the order of ± 2 per cent. This includes the operation of delivering 0.05 cc. of blood (from a calibrated pipette) into 20 cc. of diluting fluid. This degree of accuracy has been found in all of the other procedures in which there is a reliable method of establishing the true results.

Permanence of Calibration—The determinations whose results have been used in Table I have been chosen at random from our records over a period of about 2 years. There is no sign of any deterioration of performance, or of the development of systematic

TABLE I

Comparison of Hemoglobin Determinations by Van Slyke Oxygen Capacity Method and by Photoelectric Colorimeter

The original calibration of the photoelectric colorimeter was made by means of Van Slyke determinations. Results are expressed as percentages of an arbitrary normal of 20.9 cc. of oxygen per 100 cc. of blood.

Duplicate determinations were made. The figures in parentheses represent the averages.

Van Slyke			Photoelectric			Error
						<i>per cent</i>
100.2	99.6	(99.9)	100.0	101.0	(100.5)	+0.6
94.3	94.4	(94.35)	93.5	93.5	(93.5)	-0.9
88.0	87.9	(87.95)	87.0	86.5	(86.75)	-1.4
112.5	113.1	(112.8)	110.6	111.6	(111.1)	-1.5
75.3	74.5	(74.9)	74.0	75.0	(74.5)	-0.6
78.8	79.6	(79.2)	80.0	80.0	(80.0)	+1.0
67.6	68.4	(68.0)	67.5	66.5	(67.0)	-1.5
108.0	107.8	(107.9)	106.0	106.0	(106.0)	-1.8
86.3	87.3	(86.8)	86.6	87.6	(87.1)	+0.4
70.2	70.8	(70.5)	71.5	70.5	(71.0)	+0.7

errors greater than the admitted limit of ± 2 per cent. Possible causes of changes in calibration are deterioration of the lamp filament and small random changes in the spectral response of the photocell. Deterioration of the lamp is minimized by the low operating voltage; in addition, the lamp is checked against a standard lamp (never used for any other purpose) at intervals of about 3 months. Lamps are discarded as soon as deterioration is detected instead of waiting for them to burn out. The effect of small changes in photocell response can be rendered negligible by

adhering to certain definite principles in the choice of color filters. This will be dealt with fully in a future paper on the general theory of the use of filters in photoelectric colorimetry.

SUMMARY

A simple, easily constructed photoelectric colorimeter of the single photocell, direct reading type has been described. In this instrument, exceptional stability is secured by using a lamp of such low power requirement that it may be operated by a storage battery.

High illuminating efficiency obtained by the use of a reflector and smooth control of light intensity over a wide range permit the use of color filters of very high selectivity, thus greatly extending the scope of the apparatus.

In addition to the usual advantages inherent in the objective type of colorimeter, simplicity and convenience of operation have been improved by using standard test-tubes in place of conventional absorption cells.

Complete mechanical rigidity, absence of moving parts, and a large safety factor in all important components eliminate the usual causes of unsatisfactory performance.

The instrument has been used in the development of a number of new techniques involving analysis of mixtures of colored substances in solution. These will shortly be reported in detail.

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ON BLOOD FIBRIN

A CONTRIBUTION TO THE PROBLEM OF PROTEIN STRUCTURE

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Recently a reagent for the quantitative isolation of proline was developed in this laboratory, and with its aid it was shown that in gelatin the amino acids, glycine, proline, and hydroxyproline, occurred in molar quantities defined by the simple numerical ratio of 6:3:2 (1). These results are reproduced in Table I, supplemented by analytical values for arginine, lysine, leucine-isoleucine, and alanine.

In view of the striking regularities noted in the case of gelatin, it became desirable to determine the validity of similar relationships in a second protein. Blood fibrin was chosen as a typical example and two commercial preparations were obtained for analysis. One was of foreign origin (Hoffmann-La Roche, Basel) and the other domestic (Eimer and Amend, New York). Only those amino acids were determined for which trustworthy quantitative methods were available. The percentage composition thus obtained is presented in Table II, and the values are compared with those resulting from previous investigations. It is of interest to note that the yields obtained in this study for proline, glutamic acid, and aspartic acid are significantly higher than those reported earlier.

The percentage composition recorded in Column 1 of Table II was recalculated on a gm. molecular basis, and is presented in this form in Table III. On examination of Column 3 of Table III, it is seen that beef fibrin produces equivalent amounts of arginine, aspartic acid, and proline on hydrolysis. This fact suggests that the formation of beef fibrin from its constituent parts follows, in one way or another, certain stoichiometrical principles. Addi-

TABLE I
Amino Acid Content of Gelatin after Hydrolysis

Amino acid	Weight (1)	Mol. wt. (2)	Gm. molecules (3)	Ratio (4)	Periodicity (5)
	<i>per cent</i>				
Glycine.....	25.5	75	0.34	24	3
Proline.....	19.7	115	0.17	12	6
Hydroxyproline.....	14.4	131	0.11	8	9
Arginine (2)*.....	9.1	174	0.052	4	18
Alanine (3).....	8.7	89	0.098	8	9
Leucine-isoleucine (3).....	7.1	131	0.054	4	18
Lvsine (3).....	5.9	146	0.040	3	24

* The figures in parentheses represent bibliographic references.

TABLE II
Amino Acid Content of Cattle Fibrin after Hydrolysis (Weight in Per Cent)

Amino acid	Estimations reported in this paper (1)	Estimations reported in literature		
		By isolation (2)	By Van Slyke method (3)	Colorimetric (4)
Arginine	7.4	7.0 (4)	7.9 (6)*	7.6 (9)
	7.7	4.6 (5)	7.6 (7)	
	7.4		7.8 (8)	
Histidine	2.4	2.1 (5)	3.2 (7)	3.4 (10)
	2.5		2.8 (8)	2.2 (11)
Lysine	10.1	5.2 (2)	10.6 (7)	
			13.0 (8)	
Glutamic acid	13.8	6.6 (12)		
	14.1	10.4 (13)		
Aspartic acid	5.9	1.7 (12)		
	4.8	2.0 (13)		
Proline	4.7	2.4 (12)		
	5.1	3.6 (13)		
	4.9			

* This value was obtained by the arginase method.

tional evidence supporting this contention is found on further consideration of Columns 3 and 5¹ of Table III.

¹ The ratios in Column 5 were obtained from the experimental values presented in Column 3.

The ratios given in Column 5 (Table III) were converted into gm. molecules through a simple calculation with the value 0.0443 (gm. molecules of arginine, aspartic acid, and proline) as a base. The calculated values are to be found in Column 4, and it is evident that the difference between found and calculated values is in no case more than 5 per cent. In view of the numerous manipulations that are associated with each individual analysis, the agreement noted can be considered as satisfactory.

Impressive stoichiometrical relationships have now been encountered in the case of two separate proteins, and it is obvious

TABLE III
Ratio of Amino Acids in Blood Fibrin after Hydrolysis

Amino acid	Weight	Mol. wt.	Gm. molecules		Ratio	Periodicity
			Found	Calculated		
	(1)	(2)	(3)	(4)	(5)	(6)
	<i>per cent</i>					
Glutamic acid.....	14.1	147	0.095 ₉	0.099 ₈	72	8
Lysine.....	10.1	146	0.069 ₁	0.066 ₄	48	12
Arginine.....	7.7	174	0.044 ₂	0.044 ₂	32	18
Aspartic acid.....	5.9	133	0.044 ₂	0.044 ₂	32	18
Proline.....	5.1	115	0.044 ₂	0.044 ₂	32	18
Tryptophane (14)*.....	5.0	204	0.024 ₂	0.024 ₂	18	32
Histidine.....	2.5	155	0.016 ₁	0.016 ₈	12	48
Methionine (15).....	2.6	149	0.017 ₄	0.016 ₈	12	48
Cystine (16).....	1.5	240	0.006 ₂	0.006 ₂	9†	64†

* The figures in parentheses represent bibliographic references.

† Calculated as cysteine.

that these findings must have a rôle in the definition of the structure of the protein molecule. The simplest explanation lies in the assumption that the structural units of fibrin and gelatin are periodically arranged within the peptide chain and that each unit exhibits its own particular periodicity. On the basis of this assumption the periodicities of the various units (amino acid residues) contained in fibrin were calculated and are recorded in Table III, Column 6. The method of calculation can best be illustrated by the following example. Arginine and glutamic acid are present in fibrin in the ratio of 32:72 and it follows that their

periodicities can be expressed as one of several inverted ratios, *e.g.* 9:4, 18:8, 36:16, 72:32, etc. Therefore, considering the periodicity of glutamic acid alone, one finds the values 4, 8, 16, 32, etc. are mathematically possible. The question as to which of the above values is the correct one, when all of the other amino acids are considered, can be decided with the aid of the following equation.

$$\frac{P \times AW}{MW} = \frac{100}{\%}$$

where P = periodicity of respective amino acid

AW = average residue weight of all amino acids

MW = molecular weight of respective amino acid

$\%$ = percentage of respective amino acid in hydrolysate

With P equal to 4, 8, and 16, the calculated values for AW are 260, 130, and 65 respectively. It is at once evident that the periodicity of glutamic acid in cattle fibrin can only be 8; that is, in every molecule of this protein each glutamic acid residue is separated from the previous and succeeding ones by seven other amino acids. It should be pointed out that, regardless of the validity of the periodicity hypothesis, the fact remains that one-eighth of the amino acid residues present in beef fibrin appears as glutamic acid upon hydrolysis of the protein.

On examination of the periodicities of the various amino acids presented in Column 6 of Table III, it was noted that all of the values can be considered as members of one of two arithmetical series, *e.g.*

$$(a) \quad 1 \times 2^3; 4 \times 2^3; 6 \times 2^3; 8 \times 2^3$$

$$(b) \quad 4 \times 3; 6 \times 3; 16 \times 3$$

Similarly, in the case of gelatin (Table I) the periodicities were again members of a simple series.

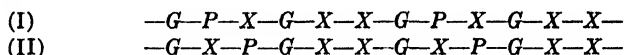
$$(c) \quad 1 \times 3; 2 \times 3; 3 \times 3; 6 \times 3; 8 \times 3$$

It is apparent that these expressions are derived from the prime numbers 2 and 3. This fact may be of significance in relation to the ability of these enormous protein molecules to assume a crystal structure. In considering the formation of fibrin and collagen *in vivo*, it is apparent that the natural growth processes must provide

a mechanism capable of making a precise selection among the available structural units so that the complicated stoichiometrical pattern with many superimposed periodicities may be obtained. Here, indeed, is an amazing example of a very complex specificity phenomenon.

The preceding paragraphs have been restricted to a discussion of two proteins, namely fibrin and gelatin. However, on examining the literature pertinent to the analysis of proteins, one can find further suggestion of valid stoichiometrical relationships. In this connection the observations of Vickery and of Block are perhaps the most extensive (17-20). These authors, studying the basic amino acids, have encountered striking ratios in the case of mammalian hemoglobins, avian and mammalian orosins, and keratins.

A short time ago, in addition to introducing new methods for the isolation of proline and glycine, two possible formulas for gelatin were advanced (1), *e.g.*



Furthermore, it was indicated that it was possible to differentiate between these two formulas through the isolation of large quantities of peptides containing glycine and proline and the determination of the sequence of the amino acids in the isolated peptides. Through the application of the kylene procedure of Siegfried, Grassmann and Riederle (21) isolated a tripeptide from gelatin and, with the aid of the new proline reagent, characterized it as lysylprolylglycine. This finding appears to support Formula II.

EXPERIMENTAL

Preparation of Fibrin Hydrolysate—300 gm. of commercial fibrin (Eimer and Amend or Hoffmann-La Roche)² were refluxed with 1500 cc. of 20 per cent hydrochloric acid for 15 hours. After cooling, the acid-insoluble humin was removed, washed with cold water, and the combined filtrate and washings were concentrated to a thick syrup. This residue was repeatedly taken up in water and evaporated, until the major portion of the hydrochloric acid had been volatilized. The hydrolysate was then made up to 1

² Both of these fibrins were prepared from beef blood.

liter. When prepared from the Eimer and Amend fibrin, 100 cc. of this solution were equivalent to 25.65 gm. of moisture- and ash-free protein.³ In the case of the Hoffmann-La Roche sample, the same volume contained 23.00 gm. (corrected).

l-Proline—200 cc. of fibrin hydrolysate (Eimer and Amend) were diluted to 1 liter and 20 cc. of a 10 per cent solution of phosphotungstic acid added under vigorous stirring. The insoluble fraction was removed by filtration and the light yellow filtrate concentrated to 170 cc. To this solution were added 16.0 gm. of recrystallized ammonium rhodanilate in 102 cc. of methyl alcohol, and the mixture was shaken on the machine for 24 hours. After standing overnight in the cold room, the homogeneous crystalline precipitate was collected and dried in the usual manner (1). The proline rhodanilate thus obtained weighed 12.0 gm. and possessed the correct composition.

$(C_5H_{14}N_6S_4Cr) \cdot (C_5H_{10}O_2N) \cdot H_2O$.	Calculated.	C 41.7, H 4.3, N 16.2
	Found.	" 41.5, " 4.4, " 16.0

Corrected for the minimum solubility of proline rhodanilate in the mother liquor (*e.g.*, 5 per cent (1)), the yield was raised to 12.6 gm., which is equivalent to 2.4 gm. of proline or 4.7 per cent of the hydrolyzed protein. A second estimation gave 5.1 per cent, and from the Hoffmann-La Roche sample 4.9 per cent was obtained.

l-Histidine—200 cc. of fibrin hydrolysate (Eimer and Amend) were diluted to 1 liter and the residual hydrochloric acid was removed with silver oxide in the presence of sufficient sulfuric acid to maintain the solution strongly acid to Congo red. After a thorough washing of the precipitated silver chloride, the filtrate and washings were concentrated to a volume of 1 liter and the histidine was separated through the use of the silver-baryta procedure, as described by Vickery and Block (23). By this method 5.80 gm. of histidine diflavinate of the following composition were obtained.

$2(C_{10}H_8N_2SO_3) \cdot (C_6H_7N_3O_2) \cdot H_2O$.	Calculated, N 12.2; found, N 12.1
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When converted to histidine, the yield is 1.12 gm. or 2.2 per cent of the hydrolyzed protein. According to Vickery and Leavenworth

³ Nitrogen content equals 17.7 per cent (22).

(24), one may expect to recover approximately 90 per cent of the histidine present when working with quantities of protein as employed above. Therefore, the corrected histidine content becomes 2.4 per cent. A similar experiment with Hoffmann-La Roche fibrin yielded 2.5 per cent (corrected) histidine.

d-Arginine—The arginine fraction resulting from the histidine estimation (Eimer and Amend fibrin) was treated according to Vickery and Block (23) and after drying at 105°, 10.0 gm. of arginine flavinate were obtained.

$(C_{10}H_8N_2SO_8) \cdot (C_6H_{14}O_2N_4)$. Calculated, N 17.2; found, N 17.0

When converted to arginine, the yield is 3.57 gm.; and after correction for the solubility of arginine silver in 6000 cc. of solution, *e.g.* 0.22 gm. of arginine (19), the yield becomes 3.79 gm. or 7.4 per cent of the original protein. As a check on the above value, 100 cc. of hydrolysate (Eimer and Amend) were diluted to 1 liter, and the solution was adjusted to pH 3.5 by the cautious addition of silver oxide. After removing and washing the precipitated silver chloride, the solution was heated to 90° and treated with 100 cc. of water containing 8.0 gm. of flavianic acid. After standing in the cold room for 3 days, the arginine flavinate was collected, recrystallized as directed by Kossel and Gross (2), and dried at 105°. The yield of arginine flavinate under these conditions was 5.5 gm., which is equivalent to 7.7 per cent arginine.

$(C_{10}H_8N_2SO_8) \cdot (C_6H_{14}O_2N_4)$. Calculated, N 17.2; found, N 17.0

A similar determination, with Hoffmann-La Roche fibrin, gave 7.4 per cent arginine.

d-Lysine—The lysine fraction resulting from the estimation of histidine and arginine by the silver-baryta method (1) was subjected to the procedure of Vickery and Block (23) as modified by Block (19). From 200 cc. of original hydrolysate (Eimer and Amend) 11.5 gm. of lysine picrate (m.p. 260° with decomposition) of the following composition were obtained.

$(C_8H_8O_7N_2) \cdot (C_6H_{14}O_2N_2)$. Calculated, N 18.7; found, N 18.8

When converted to lysine, the yield is 4.48 gm., to which must be added 0.23 gm. as a solubility correction arising from the solubility of lysine phosphotungstate (25), giving a total yield of 4.71 gm.

or 9.2 per cent of the hydrolyzed protein. Block (25), citing unpublished experiments, states that under the above conditions the yield of lysine, corrected for the solubility of lysine phosphotungstate, is about 90 per cent of the amount actually present. Allowing for this additional correction, the amount of lysine in the fibrin hydrolysate is raised to 10.1 per cent.

d-Glutamic Acid—200 cc. of hydrolysate (Eimer and Amend) were diluted to 2 liters and the basic amino acids removed with phosphotungstic acid as directed by Van Slyke (7, 26). The filtrate from the phosphotungstic acid precipitation was freed of excess reagent by extraction with amyl alcohol and ether, and the resulting solution of monoamino acids evaporated to a thick syrup. The syrupy residue was taken up in 300 cc. of water and the glutamic and aspartic acids were isolated as their barium salts, according to the procedure described by Jones and Moeller (27). On decomposition of the barium salts, 4.5 gm. of glutamic acid were isolated as the hydrochloride (5.6 gm.).

$C_5H_{10}O_4NCl$. Calculated, N 7.6; found, N 7.6

From the mother liquors after the separation of aspartic acid an additional 2.6 gm. of glutamic acid were obtained, making the total yield 7.1 gm. or 13.8 per cent of the hydrolyzed protein. The yield from the Hoffmann-La Roche sample was 14.1 per cent.

l-Aspartic Acid—The filtrate and washings resulting from the separation of glutamic acid hydrochloride (above) were treated as directed by Jones and Moeller (27),⁴ and the aspartic acid was isolated in the form of its insoluble copper salt. From 200 cc. of original hydrolysate (Eimer and Amend) 6.1 gm. of copper aspartate were obtained.

$C_4H_5O_4NCu \cdot 4H_2O$. Calculated. N 5.3, Cu 23.7
Found. " 5.3, " 24.5

When converted to aspartic acid, the yield is 3.05 gm. or 5.9 per cent. In the case of the Hoffmann-La Roche sample, the yield was 4.8 per cent, but this low value was associated with known manipulative losses.

⁴ On p. 435, under the title "Separation of aspartic acid," the obvious step of removing the excess silver and sulfate ion after the precipitation of hydrochloric acid with excess silver sulfate is not mentioned.

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EFFECT OF SHORT ELECTRIC WAVES ON STEROL COLLOIDS

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In recent years numerous investigations have been concerned with the effects of short electric waves on biological materials. Almost exclusively they deal with the heat effect, which, in our opinion, is an indirect one and is certainly not distinctive for electric waves. With the exception of some scattered observations (1, 2), the question of a specific effect, aside from the heat effect, was left open.

In order to detect such an effect it was necessary to study the conditions for accurate temperature regulation, and to find a system sufficiently unstable to respond to a small stimulus. A fair degree of temperature control had been attained by Hicks and Szymanowski (1). We followed their technique in large measure. It was more difficult to find a colloid suitable for such experiments, but a whole set of such colloids was found in cholesterol and cholesterol esters, which show interesting instabilities with age and are at the same time heat-stable. The stability to heat was a particularly fortunate feature because it helped to eliminate all doubt as to residual fluctuations that are present even in the most accurate temperature regulation. Since the effect observed was an accelerated coarsening of the colloid, we measured the coarsening by means of a nephelometer. It was necessary to run strict blanks for each condition and to restrict the work to practically one wave-length, 4.8 meters, and one strength of colloid, namely 0.3 mg. per cent.

EXPERIMENTAL

Preparation of Colloids—The colloids were prepared by adding 1 per cent acetone solutions of cholesterol or cholesterol acetate

to boiling distilled water, and continuing the boiling till the last traces of acetone were gone. The strength was adjusted to 0.3 mg. per cent by addition of water after filtration through sintered glass filters. The sterol or sterol ester used was rendered free of oxidation products by repeated recrystallizations from acetone. The oxidation products tend to stabilize the colloid unduly.

Operation of Short Wave Generator—The generator was a variable wave-length oscillator with an inductively coupled resonating

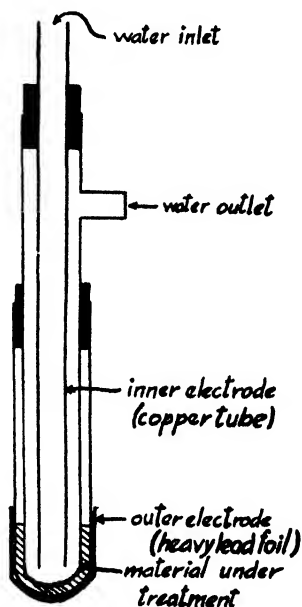


FIG. 1. Wave exposure apparatus. The outer glass tube measures 7×2 cm.; the other parts to scale.

circuit made especially for this work and loaned through the generosity of the Westinghouse X-Ray Corporation. As this particular machine gave a maximum output at a wave-length of 4.8 meters, the work was done at that wave-length principally. As the output in the condenser field and the transfer of energy between the condenser plates is affected considerably by leakage through moisture around the outside of the cooling system, the output regulation was difficult, especially in damp weather, and

had to be watched constantly. The output in these experiments ranged from 400 to 490 milliamperes.

Wave Exposure Apparatus—Fig. 1 shows the set-up.

TABLE I
Coarsening of Sterol Colloids on Irradiation with Short Electric Waves

Colloid	Input	Time	Wave-length	Temperature	Age before irradiation	Shutter openings of nephelometer with unirradiated standard at arbitrary scale divisions of			
						5.0	10.0	15.0	20.0
	milli-amperes	hrs.	meters	°C.	days				
Cholesterol 1	420	1	4.8	15.3	18	6.2	12.2	18.1	23.9
	420	1	4.8	15.0	18	6.1	12.1	18.2	24.1
	420	1	4.8	14.8	18	6.1	12.2	18.0	23.7
Average.....						6.1	12.2	18.1	23.9
Cholesterol 2	400	1	4.8	15.4	9	5.3	11.4	17.1	
	420	1	4.8	17.4	13	5.5	12.2	18.1	
	420	1	4.8	15.0	17	6.0	12.4	19.1	
Cholesterol Acetate 1	420	1	4.8	15.0	5	5.0	10.4	15.9	
	420	1	4.8	15.0	7	5.0	10.7	16.2	
	400	1	4.8	13.5	21	5.7	10.9	15.9	
Cholesterol Acetate 2	420	1	4.8	13.8	21	5.6	10.9	15.9	
	420	1	4.8	15.5	4	5.1	10.2	15.5	
	430	1	5.5	16.0	4	5.2	10.2	15.3	
	450	1	5.5	15.0	22	5.5	10.9	16.9	
	410	1	4.8	14.7	20	5.3	10.6	16.7	
	420	1	4.8	14.8	22	5.3	10.7	16.7	
	420	1	4.8	13.5	44	5.7	11.5	18.4	
	430	2	4.8	14.1	26	5.4	10.8	16.7	
	430	7	4.8	15.0	22	5.6	11.5	17.9	24.8
	490	11.5	4.8	15.0	11	5.4	11.1	16.8	22.4

Nephelometer—The instrument used was an Ernst Leitz Wetzlar No. 2559. At least twenty readings were taken for each solution at four different settings.

Procedure

A colloid is introduced into the apparatus, and is then irradiated by the waves for a definite span of time. Another sample is

exposed to the same conditions without irradiation. The two solutions are compared in the nephelometer at definite time intervals. As a blank the unirradiated solution is compared with itself as a check on the settings of the apparatus.

The data are summarized in Table I. Two cholesterol and two cholesterol acetate colloids were used. In two cases the wavelength was varied to 5.5 meters. The time of operation has been kept at 1 hour, since the apparatus operates badly after $1\frac{1}{2}$ hours. In the cases in which the exposures were more than an hour, the result was obtained by the addition of runs lasting 1 to $1\frac{1}{2}$ hours.

Special experiments were run to determine the effect of temperature on the stability of the colloid. No difference was noted in the nephelometer between colloids kept for an hour near boiling, overnight in the ice box, and at ordinary room temperature.

Samples were also kept in different containers, such as soft glass and Pyrex flasks and measuring tubes, without effect on the nephelometer readings.

DISCUSSION

Dilution of a typical cholesterol colloid of the strength used in these experiments shifts the nephelometer shutter opening readings to higher values. At half strength these rise to 7.9, 16.7, and 25.4 for the corresponding full strength readings of 5.0, 10.0, and 15.0. We have therefore observed an effect akin to the effect of dilution, a decrease of surface, which we can attribute most plausibly to a coarsening of the colloid treated with the short electric waves. Our largest effect corresponds to a reduction of material of about 25 per cent.

Our chief concern has been in making sure that the effect is positive and reproducible. The results were always positive. A high degree of reproducibility is indicated in the experiments on Cholesterol 1, for which the results have been averaged in Table I.

Definite trends are observable. The effect is increased with the age of the colloid, as particularly shown by the experiments with Cholesterol 2. This colloid, however, was more stable than Cholesterol 1, the cholesterol being of somewhat lower melting point owing to impurities. The results are not as clear cut for a still more stable colloid, as Cholesterol Acetate 1 turned out to be. The results again are a little clearer in the case of Cholesterol

Acetate 2 for both wave-lengths. There seems to be no definite effect that can be attributed to a change of wave-length for the two tried. The effect of time of exposure is also definite but not strongly marked, the age of the colloid being a much more important factor. All these observations, obviously, are in line with the supposition that a coarsening of the particles takes place. One would expect the clumping to show in a more marked fashion with older colloids, and to increase with time of exposure. Since the effect is visioned to be a physical one, rather than a chemical one, because of the low quantum of energy in the radiation, one would not expect a marked effect due to change in wave-length.

SUMMARY

Short electric waves of the order of 5 meters have a coarsening effect on aging cholesterol and cholesterol acetate colloids. The effect is independent of temperature and shows a tendency to increase with the age of the colloid and the length of time of exposure.

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**THE SYNTHESIS OF *d*-CARNOSINE, THE ENANTIO-
MORPH OF THE NATURALLY OCCURRING FORM,
AND A STUDY OF ITS DEPRESSOR EFFECT ON
THE BLOOD PRESSURE**

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The question of the relationship of spatial configuration to physiological activity has been the subject of a considerable number of investigations and many instances of specificity with regard to spatial configuration have been found. One need only recall the very striking difference in the effect on the blood pressure of levorotatory and dextrorotatory adrenalin, the former being 15 times as active as the latter (1), to realize what differences can be found in the pharmacological action of optical isomers.

Although numerous studies of such a nature have been carried out on pressor substances, surprisingly little work has been done from this standpoint on depressor compounds. In fact von Braun and Jacob (2) stated as recently as 1933 that no study of the influence of configuration upon the pharmacological effect of blood pressure-lowering substances had, to the best of their knowledge, been made up to that time. These investigators therefore undertook the study of the action of *d*- and *l*-fenchelylisocyanate derivative of choline and found that both isomers exerted about the same degree of depressor activity. On the smooth muscle of the earthworm, however, the *d* derivative was 50 times as active an excitant as the levorotatory isomer. Recently Major and Bonnett (3) succeeded in preparing the optical isomers of acetyl- β -methylcholine and studied their muscarine-like action on the blood pressure. They observed that acetyl-*l*- β -methylcholine was approximately 100 times less active than the racemic isomer. Of course, the results of these two investigations are not necessarily

comparable, since in the fenchyl compound the asymmetric carbon is in the acyl group, whereas in the latter case it is present in the choline portion of the molecule. Aside from these two studies we know of no others on the relationship of spatial configuration to depressor activity. We therefore became interested in whether specificity with regard to spatial configuration existed in the case of the blood pressure-lowering dipeptide carnosine, β -alanyl-*l*-histidine, which occurs in muscle tissue.

The fact that carnosine is a dipeptide consisting of an optically inactive β -amino acid attached to an optically active amino acid, *l*-histidine, attaches particular interest to the pharmacological activity of its enantiomorph. Since both *d*- and *l*-histidine appear to be utilizable physiologically for growth purposes (4), it might be expected at first sight that both isomers linked with β -alanine would be pharmacologically active. On the other hand, we have found in the case of tryptophane that, although both *d*- and *l*-tryptophane are physiologically utilizable, the acetyl derivative of the *d*-tryptophane, the foreign isomer, is not utilized, in contrast to the acetyl-*l*-tryptophane (5). From this standpoint one might surmise that the β -alanyl derivative of *d*-histidine might have little or no action. From still another standpoint it might be expected that the *d*-carnosine might even be more active than the *l*-carnosine. If the transitory nature of the action of *l*-carnosine on the blood pressure were due to the hydrolysis of the peptide by some special dipeptidase, then it is conceivable that the unnatural isomer which might be resistant to hydrolysis might even produce a more lasting effect. A somewhat analogous situation might exist in the case of acetyl- β -methylcholine. For example, the acetyl-*l*- β -methylcholine might have intrinsically a greater effect on the blood pressure than the dextro isomer, but, being hydrolyzed more rapidly by the esterase of the blood, its total effect might be less.

In undertaking the preparation of the *d*-carnosine, we decided to prepare it by first isolating *d*-histidine and then linking it with β -alanine rather than by racemizing the *l*-carnosine and resolving the racemic isomer. The *l*-histidine monohydrochloride, isolated from dried blood corpuscles, was therefore racemized by a slight modification of the method worked out by du Vigneaud and Meyer (6) for the racemization of certain other amino acids. 1

mole of histidine monohydrochloride was treated with 20 moles of acetic anhydride and 2.2 moles of NaOH and the temperature of the reaction kept at 55° for 16 hours. The resulting acetyl-*dl*-histidine was hydrolyzed and the free *dl*-histidine was resolved with tartaric acid by the method of Pyman (7). The *d*-carnosine was then prepared by the method worked out by Sifferd and du Vigneaud (8) for *l*-carnosine. The *d*-histidine methyl ester was condensed with carbobenzoxy- β -alanine azide and the resulting carbobenzoxy- β -alanyl-*d*-histidine methyl ester was saponified and the carbobenzoxy group split off by reduction with hydrogen in the presence of palladium. The *d*-carnosine possessed a specific



FIG. 1. The left-hand section shows crystals of *l*-carnosine; the right-hand, *d*-carnosine.

rotation of $[\alpha]_D^{25} = +20.4^\circ$ and a melting point of 260° , which correspond closely to the values reported for *l*-carnosine (8); namely, a melting point of 260° and a specific rotation of $[\alpha]_D^{25} = +20.5^\circ$. The *d*-carnosine, furthermore, had the same crystalline structure as *l*-carnosine, as shown in Fig. 1. The crystalline hydrochlorides were also prepared and the two forms were found to have the same crystalline structure and melting point.

The *dl*-carnosine was prepared by mixing exactly equal amounts of the active isomers and crystallizing the mixture from water and alcohol. The *dl*-carnosine melted at 260° and appeared to have the same crystalline structure as the active forms.

The effect of *d*-carnosine and *dl*-carnosine on the blood pressure

was tested by intravenous injection of the compounds into cats under both sodium amytal and luminal anesthesia, the blood pressure being recorded in the usual manner from the carotid artery. As shown in Fig. 2, *d*-carnosine in 20 times the dose of *l*-carnosine produced no depression in the blood pressure, thus demonstrating the remarkable difference in the depressor activity of these two optical isomers. The depressor activity of the compounds was also tried on rabbits under sodium amytal. The same lack of depressor activity on the part of the dextro isomer was observed. The *dl*-carnosine, as would be expected, exerted about one-half as much activity as the naturally occurring isomer.

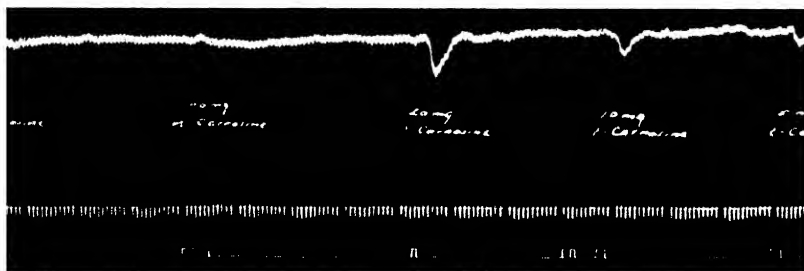


Fig. 2. Effect of *d*- and *l*-carnosine on the blood pressure of a cat under luminal anesthesia.

EXPERIMENTAL

Racemization of l-Histidine 25 gm. of *l*-histidine monohydrochloride were dissolved in 75 cc. of 3.893 *N* NaOH and 50 cc. of water. The solution was cooled to 0° in an ice bath and 250 cc. of acetic anhydride were added in small portions, with shaking and cooling. The reaction mixture was then kept at 50–55° for 16 hours, and, after the solution was cooled, 41.1 cc. of 3.919 *N* H₂SO₄ were added. The solution was concentrated *in vacuo* to a thick syrup, 50 cc. of water were added, and the mixture was again evaporated. This was repeated two more times. An oily residue remained which was extracted with 200 cc. of hot ethyl alcohol in four portions. The alcohol extracts were combined and the alcohol was removed by vacuum distillation. An oil residue remained which was taken up in 100 cc. of hot water, and 2 volumes of acetone were added. When the solution was cooled to 0°, the

acetyl-*dl*-histidine crystallized. 18.5 gm. of the material were obtained. It melted at 148° (corrected), which agrees with the value given by Bergmann and Zervas (9). The product possessed no rotation, indicating complete racemization.

In a flask fitted with a condenser connected by a ground glass joint, 18.5 gm. of acetyl-*dl*-histidine and 370 cc. of 2 *N* HCl were placed. This mixture was heated for 1 hour in an oil bath at 125–130°. After treatment of the solution with norit, the filtrate was concentrated *in vacuo*. The residue was dissolved in 600 cc. of warm ethyl alcohol and after the solution had been cooled to room temperature, 50 cc. of freshly distilled aniline were added slowly with stirring (10). After standing for 12 hours in the ice box, the solution was filtered and the precipitated *dl*-histidine monohydrochloride was washed with several portions of alcohol. After the product had been recrystallized from water by the addition of 3 volumes of alcohol, it melted at 157° (corrected). The yield was 16.3 gm.

The free histidine was obtained by means of MgO, essentially as used by Vickery and Leavenworth (11) for *l*-histidine. 16.3 gm. of *dl*-histidine monohydrochloride were dissolved in 160 cc. of water and MgO was added to this solution until the reaction of the solution was just alkaline to litmus. The solution was boiled with 0.2 gm. of norit and the mixture was filtered. The pH of the filtrate was then adjusted to 7.2 with dilute HCl and the solution was concentrated to 50 cc. 3 volumes of absolute alcohol were then added and the solution was allowed to remain in the ice box for 24 hours. The *dl*-histidine was filtered and was washed with absolute alcohol. A yield of 9.5 gm. was obtained. The mother liquor was concentrated to 20 cc. and 80 cc. of absolute alcohol added. An additional 1.3 gm. of *dl*-histidine crystallized slowly. The compound showed no optical activity.

Preparation of d-Histidine Methyl Ester Dihydrochloride—10.8 gm. of *dl*-histidine were resolved by the method of Pyman (7). The yield of the recrystallized *d*-histidine tartrate was 8.3 gm.

The *d*-histidine monohydrochloride was obtained from the tartrate by means of mercuric chloride. 8.3 gm. of *d*-histidine-*d*-tartrate were dissolved in 200 cc. of warm water and the solution treated with 25 gm. of mercuric chloride in 350 cc. of alcohol. The solution was kept just neutral or slightly alkaline during the

addition of the mercuric chloride by the addition of sodium carbonate solution. The mercury salt was filtered from the solution and washed with alcohol and water. The mercury salt was then suspended in 300 cc. of water and the solution saturated with hydrogen sulfide until the mercury was completely precipitated. The mercuric sulfide was removed by filtration and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in 25 cc. of water and 100 cc. of alcohol added. 4.9 gm. of the *d*-histidine monohydrochloride were obtained.

4.9 gm. of *d*-histidine monohydrochloride were suspended in 55 cc. of absolute methyl alcohol and dry hydrogen chloride passed into the solution until the histidine had all dissolved, the alcohol being allowed to reflux during the process. The reaction mixture was then cooled and the stream of dry HCl was continued. The *d*-histidine methyl ester crystallized as the solution cooled. After standing in the ice box for several hours, the mixture was filtered. The yield of *d*-histidine methyl ester dihydrochloride was 5.2 gm. The product melted at 197° (corrected) and had a specific rotation of $[\alpha]_D^{26} = -9.8^\circ$; this corresponds to the rotation of the levo ester which we found to have a rotation of $[\alpha]_D^{23} = +9.9^\circ$ for a 1 per cent aqueous solution. The product had the following composition.

3.261 mg. substance: 0.496 cc. N at 25.7° and 763 mm.

$C_7H_{13}O_2N_3Cl_2$. Calculated, N 17.36; found, N 17.44

Preparation of d-Carnosine—The directions of Sifferd and du Vigneaud (8) for the synthesis of carbobenzoxy-*l*-carnosine were followed for the preparation of carbobenzoxy-*d*-carnosine, the *d*-histidine methyl ester dihydrochloride being employed in place of the levo isomer. 5.6 gm. of the former were used in the synthesis and 6.4 gm. of the carbobenzoxy-*d*-carnosine were obtained. The compound was found to have the same melting point as the levo isomer, namely 161° (corrected). It possessed a rotation of $[\alpha]_D^{24} = -11.0^\circ$ for a 1 per cent aqueous solution, which agreed in magnitude with the rotation of $[\alpha]_D^{24} = +10.5^\circ$ which we found for the carbobenzoxy-*l*-carnosine. The reduction of the carbobenzoxy derivative and isolation of the *d*-carnosine were carried out as previously described for *l*-carnosine (8). From 6 gm. of carbobenzoxy-*d*-carnosine, 3.2 gm. of *d*-carnosine melting at 260° were obtained. The *d*-carnosine had a specific rotation of $[\alpha]_D^{28} =$

—20.4° for a 2 per cent aqueous solution. The compound had the following composition.

4.196 mg. substance: 7.39 cc. 0.009863 N HCl (micro-Kjeldahl)
 $C_9H_{14}O_3N_4$. Calculated, N 24.77; found, N 24.33

Preparation of d- and l-Carnosine Monohydrochlorides—1 gm. of *l*-carnosine was dissolved in 4.96 cc. of 0.8891 N HCl and 6 volumes of absolute ethyl alcohol were added slowly with stirring. The *l*-carnosine hydrochloride crystallized in rectangular plates as the solution was allowed to stand for a few hours at 0°. 1.0 gm. of the compound melting at 245° was obtained. The material, dried at 110° over P_2O_5 at 25 mm., possessed the following composition.

5.143 mg. substance: 7.93 cc. 0.009863 N HCl (micro-Kjeldahl)
 $C_9H_{13}O_3N_4Cl$. Calculated, N 21.34; found, N 21.30

The *d*-carnosine monohydrochloride was similarly prepared and found to have the same melting point and crystalline structure as the *l*-carnosine hydrochloride. After being dried *in vacuo* as above, it was found to have the following composition.

3.194 mg. substance: 4.95 cc. 0.009863 N HCl (micro-Kjeldahl)
 $C_9H_{13}O_3N_4Cl$. Calculated, N 21.34; found, N 21.41

SUMMARY

d-Carnosine has been synthesized and its effect on the blood pressure has been compared with that of the naturally occurring isomer, *l*-carnosine. When the *d*-carnosine was injected in even 20 times the dose of *l*-carnosine, no depressor action was detected, thus demonstrating the remarkable specificity of the depressor activity of *l*-carnosine with respect to spatial configuration.

dl-Carnosine was also prepared and, as would be expected, was found to possess one-half the depressor activity of *l*-carnosine.

The preparation of the monohydrochlorides of both *d*- and *l*-carnosine has been described.

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THE EFFECT OF α -AMINO ACIDS AND MAGNESIUM ON THE ACTIVITY OF KIDNEY AND INTESTINAL PHOSPHATASES

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The kidney and intestine are, together with bone, the rich sources of phosphatase in the organism (1). Since the phosphatases from these three tissues are not identical, as judged by their actions on different substrates or in the presence of various retardants (2, 3), it is necessary to determine whether the effect of α -amino acids, previously described (4) as being intimately related to the activity of the bone phosphatase, also holds for the kidney and intestinal enzymes.

It was shown (4) that α -amino acids in very low concentrations, about 0.0001 to 0.01 M, increased the activity of bone phosphatase by preventing its inactivation during the course of the reaction. Greater concentrations exerted a retardant effect. Magnesium ion was found to prevent inactivation and to increase the velocity with which the reaction started. In the presence of optimal concentrations of both magnesium and an α -amino acid, a direct proportionality was established between the reaction velocity, as measured by the reciprocal of the time necessary to effect a given liberation of inorganic phosphate, and the concentration of bone phosphatase. This was taken to indicate that, under these conditions, the concentration of active enzyme was identical with the apparent concentration.

Kidney and intestinal tissue have a much greater autolytic capacity than bone (5) and their extracts contain a greater concentration of α -amino acids. The accelerant effect of α -amino acids, if existent, would be demonstrable only in sufficiently dialyzed preparations.

In the present paper it is shown that such an accelerant effect exists and that at optimal concentrations of an α -amino acid and magnesium, the reaction velocity is proportional to the concentration of intestinal or kidney phosphatase. Since the demonstration parallels, in the main, that previously given for bone phosphatase, the relevant data are submitted with only brief comment. The rôles of the concentration of autolytic products and of dialysis in the estimation of the activity of extracts are treated more fully.

EXPERIMENTAL

The methods for preparing the enzyme extracts were similar to those described previously (4, 6, 7). External fat was first removed. The tissue, small intestine or the whole kidney, was then washed in physiological saline solution, and the intestinal contents, if present, removed. For extraction, 20 cc. of distilled water and 1 cc. of toluene were used per gm. of tissue; autolysis proceeded 2 to 3 days at room temperature. Details concerning the materials employed and the method of carrying out the hydrolytic reaction have been previously described (4, 6). Visking artificial sausage casings, 4 cm. in diameter, were employed for the dialysis of the extracts. In hydrolyses where the initial portion of the reaction was determined, the components and volume of the hydrolysis mixture and the sample taken were increased several fold to permit the determinations of the small amounts of phosphate liberated. The activity of the phosphatase is expressed as the reciprocal of the time in minutes necessary to liberate a given amount of phosphorus (usually 0.05 mg.) as inorganic phosphate per cc. of hydrolysis mixture. The reciprocal is designated as *Q*. The concentration of enzyme extract is expressed as volumes per cent of the hydrolysis mixture. All hydrolyses presented in this paper were carried out at optimal pH.

Results

In Table I are shown the effects of magnesium ion and of glycine on the early course of the hydrolysis of sodium β -glycerophosphate by dialyzed preparations of kidney and intestinal phosphatases. The rabbit intestinal phosphatase, Preparation RbIA-d, had been dialyzed about 16 hours against two changes of distilled water. The rat kidney phosphatase, Preparation RKJ-d, had been dialyzed for 29 hours against three changes of distilled water.

Magnesium ion increases the velocity with which the reaction starts. Thus in the case of the rat kidney phosphatase, Preparation RKJ-d, the *average* velocity of liberation of phosphorus as phosphate during the first 10 minutes, without any addition of magnesium or glycine, was 0.00030 mg. per cc. of hydrolysis mixture per minute. In the presence of an optimal concentration of magnesium ion, the velocity was 0.00060 mg. of phosphorus liberated per minute. The presence of glycine prevented the rate of

TABLE I

Effect of Glycine and of Magnesium on the Course of Hydrolysis of Sodium β -Glycerophosphate by Dialyzed Preparations of Kidney and Intestinal Phosphatases

Temperature, 25°; concentration of enzyme, 12.5 per cent by volume of hydrolysis mixture; concentration of sodium β -glycerophosphate, 0.0127 M; optimal pH.

Rat kidney phosphatase, Preparation RKJ-d				Rabbit intestinal phosphatase, Preparation RbIA-d			
Time	P liberated as phosphate per cc. hydrolysis mixture in presence of			Time	P liberated as phosphate per cc. hydrolysis mixture in presence of		
	No addition	0.00625 M glycine	0.009 M magnesium		No addition	0.00625 M glycine	0.007 M magnesium
min	mg.	mg.	mg.	min.	mg.	mg.	mg.
0				0			
10	0 0030	0 0030	0 0060	50	0 0024	0 0023	0 0080
20	0 0059	0 0059	0 0122	100	0 0046	0 0045	0 0160
30	0 0087	0 0087	0 0184	200	0 0082	0 0090	0 0315
50	0 0143	0 0148	0 0300	400	0 0148	0 0178	
90	0 0246	0 0268	0 0545				
120	0 0308	0 0362					

liberation from decreasing rapidly. Thus without any addition of magnesium or glycine, 0.0308 mg. of phosphorus had been liberated per cc. of hydrolysis mixture at the end of 120 minutes. In the presence of an added concentration of 0.00625-M glycine, 0.0362 mg. had been liberated at the end of this time, though the velocity initially was the same as in the hydrolysis in which no glycine had been added. Similar results were obtained with the intestinal phosphatase.

As was pointed out for bone phosphatase (4), the prevention by

glycine of the rapid decrease in the rate of hydrolysis of glycerophosphate is reflected in the reciprocal of the time in minutes necessary for the liberation of 0.05 mg. of phosphorus as phosphate per cc. of hydrolysis mixture. Table II contains typical data demonstrating this fact. The concentration at which this prevention was optimal was about 0.00625 M. Isoleucine also showed a similar effect; rabbit intestinal phosphatase, Preparation RbIA-d, was used.

TABLE II

Dependence of Activity of Dialyzed Kidney and Intestinal Phosphatases on Concentration of Glycine

Temperature, 25°; concentration of enzyme, 12.5 per cent; optimal pH.

Concentration of added glycine	$Q_{0.05}$ = reciprocal of time in min. necessary to liberate 0.05 mg. P as phosphate per cc. hydrolysis mixture			
	Rat kidney		Rat intestine	Rabbit intestine
	Preparation RKH-d	Preparation RKJ-d	Preparation RIJ-d	Preparation RbIA-d
<i>M per l.</i>				
0.0	0.00030	0.0039	0.0028	0.00095
0.00031	0.00064			
0.000625				0.00107
0.00125		0.0044		
0.0031	0.00101			
0.00625		0.0054	0.0049	0.00119
0.0156	0.00101			
0.0312	0.00088			
0.0625				0.00054

Relation between Reaction Velocity and Concentration of Kidney or Intestinal Phosphatase

In a previous paper (6) it was pointed out, as Northrop had done for pepsin (8), that autolytic products present in intestinal and kidney phosphatase preparations combined, especially at the higher concentrations of enzyme, to form enzymically inactive compounds. The active concentration was thus much less than the apparent. To illustrate from the previous paper (6), the activity, $Q_{0.05}$, of a rat intestinal phosphatase, Preparation RIA, was 0.0180 at 12.5 per cent and 0.0420 or only 2.3 times as much at a 6-fold, or 75 per cent, concentration of enzyme.

The α -amino acids, present as products of autolysis, may be removed by dialysis. When the dialysis was continued for a sufficient length of time, the concentration of α -amino acids became suboptimal. In such extracts, the reaction velocities at the low concentrations of enzyme were less than called for by direct proportionality of enzyme concentration to reaction veloci-

TABLE III

Effect of Optimal Concentrations of Magnesium, and of Magnesium and Glycine on Relationship between Reaction Velocity and Enzyme Concentration in Dialyzed Kidney and Intestinal Phosphatases

Temperature, 25°; optimal pH.

Phosphatase	Accelerant	Q, at		Q at 75 per cent Q at 12.5 per cent
		12.5 per cent phos- phatase	75 per cent phos- phatase	
Rabbit intestine, Preparation RbIA-d	None	0.00095	0.00714	7.5
	0.007 M Mg	0.00312	0.0179	5.7
	0.00625 M glycine + 0.009 M Mg	0.00328	0.0192	5.9
Rat intestine, Preparation RIJ-d	None	0.0028	0.0206	7.4
	0.009 M Mg	0.0160	0.0678	4.2
	0.00625 M glycine + 0.009 M Mg at 12.5%, 0.027 M Mg at 75% enzyme	0.0203	0.120	5.9
Rat kidney, Prep- paration RKF-d	None	0.0054	0.0357	6.6
	0.00625 M glycine + 0.009 M Mg	0.0106	0.0606	5.8
Rat kidney, Prep- paration RKJ-d	None	0.0039	0.0395	10.1
	0.00625 M glycine + 0.009 M Mg at 12.5%, 0.045 M Mg at 75% enzyme	0.0122	0.0725	5.9

ties (Table III), and were increased by the subsequent addition of an α -amino acid (Table II). It was noted that the degree of disproportionality and the extent of acceleration by the α -amino acid depended on the length of dialysis. Thus a kidney phosphatase, Preparation RKF-d, dialyzed overnight against two changes of distilled water, showed a ratio of the velocity at 75 per cent to that at 12.5 per cent of 6.6. The velocity at 12.5 per cent was increased

from 0.0054 to 0.0060 by the addition of 0.00625 M glycine. On the other hand, the kidney phosphatase, Preparation RKH-d, dialyzed for 3 days against five changes of distilled water, showed a ratio of the velocities at the two concentrations of enzyme of 15.5. The addition of glycine increased the velocity at 12.5 per cent from 0.00030 to 0.00101 (Table II).

Magnesium ion was removed fairly effectively during dialysis; subsequent addition of an optimal concentration resulted in a rise of activity of several hundred per cent (Table III). When magnesium ion and glycine were added so that their concentrations were optimal, maximal velocities were obtained and there was a direct proportionality between these velocities and the concentration of kidney or intestinal phosphatase. Thus without any addition, the dialyzed kidney phosphatase, Preparation RKJ-d, showed a reaction velocity of 0.0039 at 12.5 per cent concentration of enzyme and 0.0395 at 75 per cent; the ratio of the velocities was 10.1. In the presence of optimal concentrations of magnesium and glycine, Q was 0.0122 at 12.5 per cent and 0.0725 at 75 per cent; the ratio of the velocities, 5.9, was practically that of the concentrations.

Effect of Dialysis on Determination of Activity of Kidney and Intestinal Phosphatase

In order to demonstrate the accelerant effect of α -amino acids and the proportionality relationship described in the preceding section, it was necessary to dialyze the extracts sufficiently so that the concentration of α -amino acids, after the addition of the accelerants, would not be greater than optimal. It was noted that prolonged dialyses apparently resulted in the inactivation of the intestinal or kidney phosphatase.

The possibility of such inactivation has a practical bearing on the determination of the phosphatase activity of intestinal or kidney extracts. The following data and Fig. 1 illustrate the evaluation of this factor in the case of cat intestinal and kidney phosphatases.

The crude preparations were dialyzed in Visking artificial sausage casings, 4 cm. in diameter, against a 20-fold volume of distilled water; the water was changed at 6.5, 23, and 27 hours. The determinations of activity were made at a 12.5 per cent con-

centration of enzyme, and were corrected for changes in volume of the extract due to osmosis.

The extract of cat intestinal phosphatase, Preparation CaIA, showed a value for Q of 0.0292. The addition of 0.00625 M glycine did not change this value, indicating that the concentration of α -amino acids was at least optimal before the addition of the glycine. In the presence of an optimal concentration of mag-

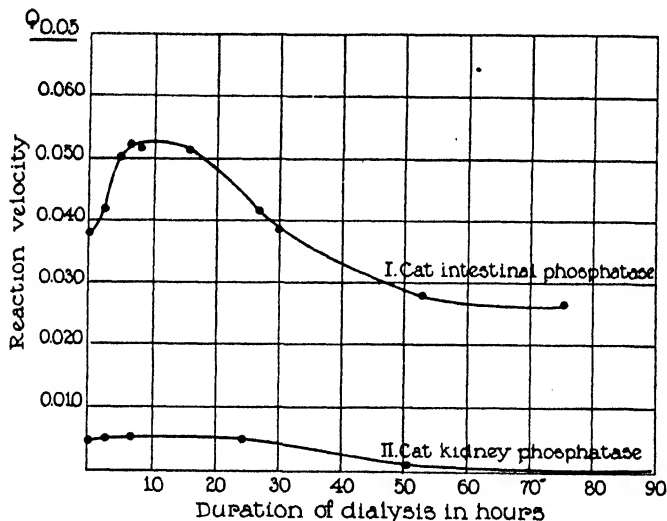


FIG. 1. Effect of dialysis at room temperature (22–25°) on the activity of cat intestinal (Preparation CaIA, Curve I) and cat kidney (Curve II) phosphatases. The tissue extract was dialyzed in Visking artificial sausage casings, 4 cm. in diameter, against a 20-fold volume of distilled water which was changed at 6.5, 23, and 27 hours. Activity was determined at 25°, at 12.5 per cent enzyme concentration, and in the presence of optimal pH and optimal concentrations of glycine and magnesium ion.

nesium, Q was 0.0380. As dialysis proceeded, Q , determined in the absence of any added accelerants, decreased from 0.0292 to 0.0281 in 6.5 hours, 0.0156 in 27 hours, and 0.0080 in 75.5 hours. This decrease indicates, in part, merely the effect due to the passage outwards of α -amino acids and magnesium and, in part, as will be seen later, the inactivation, in the later stages of the dialysis, of the enzyme. The concentration of phosphorus present as phosphate in the undialyzed and dialyzed preparations dropped

from 0.0124 mg. per cc. of hydrolysis solution to 0.0039 mg. in 2.5 hours, 0.0022 mg. in 6.5 hours, and was negligible in amount thereafter.

The reaction velocity, Q , determined in the presence of added optimal concentrations of magnesium and of glycine (Fig. 1), rose rapidly, as dialysis proceeded, to a maximal value, 0.0520, in 5 hours and remained at that value during the following 10 hours. A 1:6 dilution of a preparation dialyzed for 6.5 hours gave, at a concentration of 12.5 per cent, a proportional value for Q of 0.0088. About 15 hours after dialysis was begun, the activity began to decrease, reaching a value for Q of 0.0263 after 75.5 hours of dialysis. The water external to the dialysis bag did not show any phosphatase activity, indicating that the decrease in the activity of the extract was not due to the outward passage of any of the enzyme.

Determination of the activity of the preparations dialyzed 5 to 15 hours in the presence of optimal concentrations of magnesium and glycine and of a concentration of phosphate equal to that of the undialyzed preparation gave a value for Q of 0.0452. The difference between this value and the activity of the undialyzed extract, 0.0380, indicates the retardant effect, at this concentration of enzyme, of the α -amino acids present in the undialyzed extract. The rise in the value of Q during the first few hours of dialysis is to be attributed to the removal of retardant concentrations of α -amino acids and inorganic phosphate.

The change in the activity of the kidney phosphatase upon dialysis followed a somewhat different course. There was only a slight increase initially, indicating that the retardant effect of the concentrations of α -amino acids and phosphate in the undialyzed extract was not very marked. A precipitate, presumably of protein, appeared sometime between 8 and 24 hours after the beginning of dialysis; this was shaken with the supernatant solution when samples were taken for determination of activity. On continued dialysis beyond 24 hours, the activity began to decrease.

DISCUSSION

To the extent that the studies here reported on intestinal and kidney phosphatase have paralleled those on bone phosphatase, it would appear that the mechanism of acceleration by α -amino

acids and the concentrations at which acceleration is optimal and at which retardation occurs are similar. It has also been shown that, for the range of activities studied, the velocity of hydrolysis of sodium β -glycerophosphate is proportional to the concentration of intestinal or kidney phosphatase in the presence of optimal concentrations of magnesium ion and glycine.

However, the fact that autolyzed water extracts of kidney and intestinal tissue, in contrast to those of bone, contain a high concentration of α -amino acids as products of autolysis makes it necessary to evaluate, in a different manner, the effects of these acids in the measurement of phosphatase activity. The retardant effect, dependent on the concentration of α -amino acids, is considerable if a large concentration of extract is used, or if the autolytic capacity of the tissue of the animal used is very high.

Dialysis may be employed to eliminate the retardant effect of the α -amino acids as well as that of the inorganic phosphate. Since inactivation of the kidney or intestinal phosphatase sets in upon continued dialysis, the period of dialysis should be so chosen that the activity, determined in the presence of optimal concentrations of magnesium and glycine, is affected minimally by this inactivation and by the retardant effects of phosphate and of the products of proteolysis. In the case of the cat intestinal phosphatase, Preparation CaIA (Fig. 1), the maximal value for the activity, 0.0520, is obtained after 5 to 15 hours of dialysis.

The example of the change in activity of the cat intestinal and cat kidney phosphatases is merely illustrative of the problem of ridding the enzyme preparation of products which depress its activity. Kidney or intestinal tissue extracts of different animals may vary in their final inorganic phosphate content, in their autolytic capacity and hence the retardant effect of the α -amino acids, and finally in their susceptibility to inactivation upon dialysis.

SUMMARY

1. As in the case of bone phosphatase, α -amino acids accelerate the activity of kidney and intestinal phosphatases by preventing inactivation during the course of the reaction; magnesium increases the velocity with which the reaction starts. In the presence of optimal concentrations of glycine and magnesium, the reaction

velocity is directly proportional to the concentration of kidney or intestinal phosphatase.

2. The preceding is demonstrable in sufficiently dialyzed preparations; in direct extracts of the tissue, the concentrations of α -amino acids, because of the high autolytic capacity of kidney and intestine, are greater than optimal and retardant in effect.

3. Dialysis, designed to eliminate retardant products from extracts of kidney or intestinal phosphatase, leads, when prolonged, to inactivation of the enzyme.

4. The significance of these findings in the determination of the phosphatase activity of intestinal or kidney extracts is discussed.

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THE QUANTITIES OF PROTEIN LOST BY THE VARIOUS ORGANS AND TISSUES OF THE BODY DURING A FAST*

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The gravimetric methods we recently described (1) for the determination of the protein content of the organs and tissues of the body were used to get a direct answer to the question as to the source of the protein lost during fasting. The results enable us to allocate to each organ and tissue its absolute and relative share in the total loss of protein. The general procedure involved the measurement of the organ, tissue, and total protein of fed rats. Other rats were given nothing but water for 7 days before they were killed. On the assumption that before they were fasted they had had the same protein content as the fed rats, the difference between the total protein of the fed and fasted animals gives the total amount of protein lost, and the difference between the quantities of protein found in the organs and tissues of the fed and fasted rats gives the amount and degree to which the various parts of the body had contributed to the total loss.

In a problem of this nature, in which chemical methods are applied to biological material, the validity of the conclusions depends less on the precision of the methods of measurement than on the degree of success attained in minimizing the effect of racial and individual variation. We used a standardized albino rat derived from the Slonaker strain from which the Wistar colony was developed. For 12 years these rats had been reared under constant conditions on the same food.¹ Nevertheless, many

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¹ This is a diet recommended by Steenbock (2). It consists of corn-meal 68 gm., linseed oil cake meal 10 gm., dried ground alfalfa 2 gm., casein 10 gm., lard 5 gm., cod liver or sardine oil 3 gm., bone ash 1.5 gm., and sodium chloride 0.5 gm. It contains 18 per cent protein, 12 per cent fat, and 52 per cent carbohydrate.

anatomical and physiological measurements show that they still manifest evidence of a considerable degree of individual variation. In order to reduce the uncertainty introduced by this factor, the methods we used were designed to deal with the massed organs and carcasses derived from at least thirty individuals, and our final results are a comparison of the averages of 120 fed rats and of 120 fasted rats, derived from measurements on four separate groups of thirty fed and four groups of thirty fasted rats.

TABLE I
Protein Quantities in Gm. per Rat Found in Various Organs and Tissues in Groups of Fed and Fasted Rats

	Fed rats				Fasted rats			
	Group 1	Group 2	Group 3	Group 4	Group A	Group B	Group C	Group D
Average original live body weight, gm....	255	237	220	223	255	236	231	223
Average final live body weight, gm....	255	237	220	223	202	183	173	175
Total.....	41.8	37.0	36.3	37.1	35.4	34.1	33.7	33.1
Carcass.....	35.8	31.3	30.8		31.0	30.0	29.7	
Remaining organs.....	2.41	2.34	2.19		1.86	1.69	1.70	
Drawn blood...	1.36	1.34	1.29		1.18	1.01	1.04	
Liver.....	1.79	1.72	1.63		1.07	1.08	0.98	
Kidney.....	0.256	0.252	0.226		0.204	0.202	0.187	
Heart.....	0.141	0.135		0.128	0.119	0.107	0.108	
Testicle.....				0.222				0.225
Brain.....				0.168				0.160
Eye.....				0.0359				0.0358
Seminal vesicle.				0.0328				0.0234
Prostate.....				0.0214				0.0153
Adrenal.....				0.00377				0.00385

Male rats between the ages of 90 and 110 days were used. Two of the fasted groups were chosen so that their average original live body weights were the same as those of two of the fed groups, while there were slight differences between the average body weights of the other groups. The room in which all the groups were kept was steam-heated. There was a thermostatic control, but it was not very effective. From hourly readings of the graphic temperature records it was found that the average room temperatures for the

7 day period for the three fasted groups for which records were kept were $20.8^{\circ} \pm 1.4^{\circ}$, $20.1^{\circ} \pm 0.8^{\circ}$, and $19.6^{\circ} \pm 0.4^{\circ}$.

The actual protein measurements in the four fed and the four fasted groups are given with the original body weights in Table I.

A direct comparison of the protein quantities in fed and fasted animals can be made only between the groups of animals whose average original body weights are the same, but in all the groups we can calculate what these quantities would have been if all of them had had the same original body weight. This correction of

TABLE II

Protein Quantities in Gm. per Rat of Fed and Fasted Groups, Calculated for an Original Body Surface of 310 Sq. Cm., Corresponding to an Original Live Body Weight of 200 Gm.

	Fed rats				Fasted rats			
	Group 1	Group 2	Group 3	Group 4	Group A	Group B	Group C	Group D
Total.....	35.6	33.1	33.9	34.3	30.2	30.5	30.5	30.8
Carcass.....	30.5	27.9	28.7		26.42	26.84	26.89	
Remaining organs.....	2.06	2.09	2.04		1.58	1.51	1.54	
Drawn blood...	1.16	1.19	1.20		1.00	0.900	0.938	
Liver.....	1.52	1.53	1.52		0.911	0.965	0.885	
Kidney.....	0.218	0.225	0.212		0.173	0.180	0.169	
Heart.....	0.120	0.121		0.120	0.101	0.096	0.098	
Testicle.....				0.207				0.209
Brain.....				0.156				0.148
Eye.....				0.0334				0.0332
Seminal vesicle.				0.0305				0.0217
Prostate.....				0.0199				0.0142
Adrenal.....				0.00351				0.00357

the actual protein quantities has been made on the assumption that the protein varies as the original body surface (body surface = $9.1 \times \text{body weight}^{\frac{2}{3}}$) (3). This method was chosen because it was found that the deviation from identity was less for a body surface than for a body weight correction. The assumption is approximately valid only for rats on the same diet and of the same age and sex and is applicable not to individuals but to such averages as those with which we are here dealing. The quantities given in Table I, corrected for an original body surface of 310 sq. cm.,

equivalent to a rat of 200 gm. in body weight, are shown in Table II.

TABLE III
Protein Quantities Lost during a 7 Day Fast

The values are measured in gm. per rat with an original body surface of 310 sq. cm. (200 gm. of body weight).

Organs or tissue	Fed rats	Fasted rats	Protein lost	Proportion of total protein lost
				<i>per cent</i>
Muscle, skin, and skeleton*	28.9	26.5	2.33	62
Liver	1 53	0.92	0.607	16
Alimentary tract, pancreas, and spleen†	1.81	1.30	0.508	14
Drawn blood‡	1.19	0.95	0.240	6
Kidneys	0.218	0.174	0.0436	1
Heart	0.120	0.098	0.0217	0.6
Seminal vesicles§	0.0304	0.0217	0.0087	
Brain 	0.156	0.148	0.0080	
Prostate	0.0199	0.0142	0.0057	
Eyes	0.0333	0.0332	0.0001	
Testicles	0.206	0.209		
Adrenals	0.00350	0.00357		

* Muscle, skin, and skeleton comprise the carcass protein of Tables I and II less the protein of the brain and eyes. Since the carcass was the whole rat after exsanguination, with the heart excised and stripped of all the abdominal and pelvic organs, the term muscle, skin, and skeleton describes the nature of the great mass of tissue, though the protein of the vessels, nerves, spinal cord, and lungs is also included.

† Alimentary tract, pancreas, and spleen comprise the remaining organ protein of Tables I and II less the protein of the testicles, seminal vesicles, prostate, and adrenals. The protein of the ureters, vas deferens, epididymis, and bladder, as well as that of the pelvic and abdominal fat, was estimated with the alimentary tract, pancreas, and spleen.

‡ Drawn blood is all the blood that could be obtained from the cut abdominal aorta of the anesthetized rat while the heart was still beating.

§ Seminal vesicles do not include the protein of the contents. That fluid was removed after opening the organs by blotting between filter papers.

|| Brain is what was removed by section just below the cerebellum.

Theoretically the corrected results for the fed groups as given in Table II should have been identical, and similarly the fasted

groups should have given like results. That they are not the same may be ascribed to actual differences between groups of thirty rats of the same age and sex, to errors in measurement, and to a lack of the assumed exact correspondence between body surface and protein content under these given conditions. Actually these differences are small relative to the large differences to be observed in certain organs and tissues between any fed and any fasted group and so it is reasonable to compare the average of the fed groups with the average of the fasted groups and to regard any difference considerably greater than the differences between the component members of these averages as an effect produced by fasting. This comparison is presented in Table III.

TABLE IV
Relative Loss of Protein from Various Organs and Tissues during a 7 Day Fast

Organ or tissue	Proportion of original organ protein lost	Organ or tissue	Proportion of original organ protein lost
	<i>per cent</i>		<i>per cent</i>
Liver	40	Heart	18
Prostate	29	Muscle, skin, and skeleton ..	8
Seminal vesicles	29	Brain	5
Alimentary tract, etc.	28	Eyes	0
Kidneys	20	Testicles	0
Drawn blood	20	Adrenals	0

Table III is arranged in the order of magnitude of the absolute quantities of protein lost during the fast as given by the difference between the averages for fed and fasted animals. It will be noted that certain organs, as for instance the liver, contribute appreciable proportions of the total loss, while other organs lose no protein at all. Over 60 per cent of the total protein lost comes from the muscle, skin, and skeleton, 30 per cent is derived from the liver and alimentary tract, etc., while the remaining 8 per cent comes from all the other organs that lose protein.

In Table IV the organs and tissues are arranged in correspondence with the proportion of their original protein content lost during the fast. Table IV directly contradicts the teleological

notion that during fasting the organs most essential for life are spared, while less important tissues are depleted, for it is shown that the liver, heart, and kidneys lose relatively more of their protein than the muscle, skin, and skeleton. In fact, no single hypothesis seems adequate to explain the diversity in the proportions of protein lost. The results indicate that several mechanisms may be involved. The quite outstanding loss of protein from the liver suggests that it may be a storage depot for use in time of need, the loss from the alimentary tract, heart, and kidneys may be due to atrophy through decrease in function, while the contrast between the considerable loss from the prostate and seminal vesicles with the absence of all loss from the testicles and adrenals would seem to require an explanation in terms of a hormone mechanism.

SUMMARY

1. The source of the total protein lost by albino rats during a 7 day fast was determined by measurements of the protein content of the various organs and tissues. It was found that the muscle, skin, and skeleton contributed 62 per cent of the total, the liver 16 per cent, the alimentary tract, pancreas, and spleen 14 per cent, the drawn blood 6 per cent, and the kidneys and heart 1 and 0.5 per cent respectively. The remaining 0.5 per cent was derived from other organs. There was no loss of protein from the eyes, the testicles, and the adrenals.

2. The proportion of their original protein content lost by various organs was widely different. The liver lost 40 per cent of its protein. The prostate and seminal vesicles lost 29 per cent, while the testicles and adrenals did not lose any protein. The heart, kidneys, drawn blood, and alimentary tract lost from 18 to 28 per cent, the muscle, skin, and skeleton 8 per cent, and the brain 5 per cent.

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PROTEIN LOSS FROM LIVER DURING A TWO DAY FAST*

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After a 7 day fast the liver loses 40 per cent of its original protein content, the kidney 20 per cent, the heart 18 per cent, and all the other organs and tissues combined lose 10 per cent.¹ The fact that the liver loses so much more than any other organ suggests that it may be a depot for stored protein and that this special sort of protein may be used during fasting in much the same manner as glycogen is used during a fast. If this be the case, one would

TABLE I
Relative Loss of Organ Protein during a 2 Day Fast

Protein from	Fed rat	2 day fast	Proportion of original protein lost
	gm. per rat	gm. per rat	per cent
Liver	1.52	1.21	20
Heart	0.120	0.116	4
Kidney	0.218	0.210	4
All other organs and tissues	32.3	30.9	4

expect to find that this supposed store of protein would be drawn upon in the early stages of a fast before atrophy from decreased function or changes in the supply of hormones had led to any considerable loss from other organs and tissues. From the liver there would then occur during a fast an earlier and more pronounced loss of protein than from any other part of the body.

In order to test this hypothesis two groups of thirty male rats between 90 and 110 days of age were fasted for 2 days and their total

* This work was aided by a grant from the Rockefeller Foundation.

¹ Addis, T., Poo, L. J., and Lew, W., *J. Biol. Chem.*, **115**, 111 (1936).

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protein and the protein of the liver, heart, and kidney were determined. These groups averaged 221 and 215 gm. of live body weight. The results, calculated, as in the preceding paper, for a body surface of 310 sq. cm. (200 gm. of body weight), are given in Table I.

SUMMARY

1. After a 2 day fast the livers of albino rats lose 20 per cent of their original protein content. The kidney, the heart, and all other organs and tissues combined lose 4 per cent.

2. This pronounced loss of protein from the liver in the early stages of a fast is in conformity with the hypothesis that the livers of well fed rats contain a store of protein for use in time of need.

THE FORMATION OF VITAMIN D BY CATHODE RAYS

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The production of antirachitic substances through the agency of high speed electrons or cathode rays has been studied by Knudson and Coolidge (1), Busse (2), Knudson (3), and Knudson and Moore (4).

In the present communication, a study has been made of certain factors important in the formation of vitamin D by cathode rays. The absorption spectrum, the effect of film thickness, the time-activation curve, the effect of the ultraviolet light and x-rays accompanying the bombardment by electrons, and other variables have been considered. A kinetic treatment of the synthesis is suggested and the efficiency per electron is calculated.

EXPERIMENTAL

Procedure

A 300,000 volt cathode ray tube (5) was used. The power supply consisted of two 120,000 volt transformers used in series. The output was controlled by an autotransformer and a rheostat in the primary circuit. Although the cathode ray tube was operated at a peak voltage of 180,000, the effective voltage was much lower, since alternating current was used with mechanical rectification. Furthermore, a loss of about 70,000 volts occurs in driving the electrons through the nickel window. The average voltage of the electrons on leaving the nickel window was then about 77,000. The tube was evacuated continuously by a mercury vapor pump backed by a Cenco Megavac oil pump. A liquid air trap prevented mercury from entering the tube. The current across the cathode ray tube was 0.7 milliampere.

The ergosterol used was a preparation manufactured from molds. Assays of activated material were made by the Johns

Hopkins line test under the direction of Dr. Henry T. Scott. Each assay was made by feeding the material at one or more levels and using three rats on each level. Over 300 animals were used for these assays.

Results

Effect of Film Thickness—It is important to use very thin, uniform layers of ergosterol, since cathode rays penetrate but a short distance in solids. Otherwise the vitamin D produced will be diluted by the presence of unchanged ergosterol in the deeper layers and the potency of the sample as a whole will be low. On the other hand, extremely thin layers should be avoided because many electrons penetrate the film and lose their energy to the dish in the form of heat. This results in a higher potency of product but in a lower number of total rat units for a given time of operation. The number of electronic impacts decreases with depth. This is especially true for a tube operating with pulsating, mechanically rectified current. Electrons are emitted with a range of speeds varying from 0 voltage up to the peak voltage, and accordingly they penetrate to varying depths.

Films produced by the evaporation of ether solutions were found to be composed of small crystals and thin spots. Better films were prepared by dissolving ergosterol in acetone, then precipitating with a few drops of water. The most homogeneous and reproducible films were produced by sublimation, heating the ergosterol in a vacuum desiccator, and quickly condensing the material on a cold metal surface just above the heated ergosterol. This method was used in most cases.

Absorption Spectra—The absorption spectra of ether solutions of ergosterol exposed to cathode rays were determined by Dr. R. W. Haman, and details of this investigation will be published elsewhere.

Two interesting facts were found in this connection. The absorption spectrum differs from that of the product exposed to ultraviolet light. This seems to be another indication of the impossibility of spectroscopic analysis for vitamin D in the presence of decomposition products. The second interesting fact is the behavior at short exposures. The entire characteristic absorption of ergosterol is increased as if the concentration had

been increased. This unexpected result has not been generally reported in experiments with ultraviolet light and ergosterol, but it was found by Reerink and van Wijk (6). The increase may be due to the formation of an intermediate product or decomposition product having an absorption spectrum similar to ergosterol but with a greater absorption coefficient. This situation suggests that even the determination of unchanged ergosterol by spectrometry may be an unsound procedure. The important anomaly described here seems to be related to several other phenomena which are discussed later.

TABLE I
Activation of Ergosterol with Cathode Rays

Time	Potency, experimental	Total rat units, experimental	Potency, calculated
<i>sec.</i>	<i>rat units per mg.</i>		<i>rat units per mg.</i>
0	0	0	0
5	0.2*	4.8	1
10	0.2	4.8	2
30	0.2	4.8	5
100	7*	168	12
400	20	480	20
1000	17*	408	16
2000	10	240	10

* Potencies were estimated on the assumption that a narrow continuous line, 1+, is equivalent to 1 Steenbock unit; that a medium line, 2+, is equivalent to 5 units; and that a broad line, 3+, is equivalent to 10 units.

Time-Activation Curve—The influence of time of exposure on the activation of ergosterol by cathode rays is given in Table I. In all these cases 24 mg. of ergosterol were spread in a thin, uniform film in a nickel dish 8 cm. in diameter, placed 2 cm. below the nickel window of the cathode ray tube. The values in the last column were calculated by combining three different reactions as described later. The potencies are expressed in Steenbock units per mg.

Activation in Vacuum—Oxygen seems to have little effect on the activation for times greater than 100 seconds. At 420 seconds, for example, experiments were carried out in a nickel cell with air present and then again after evacuation with activated charcoal in a tube immersed in liquid air. In the absence of air the potency

was very slightly greater, but the difference is probably within experimental error. In another series of experiments the cell was furnished with a thin nickel window supported on a grid and was evacuated first with an oil pump and then with charcoal immersed in liquid air. The window cut down the transmission of electrons about 17 times, as determined by the decomposition of solid potassium nitrate. If the exposure times for the cell are divided by 17, the results approach the curve for direct exposure in air for times longer than about 100 seconds, but show greater potencies for the same energy at the shorter times. These results may suggest that although air has no influence on the longer exposure, oxygen may be a factor at the beginning of the process where, as stated previously, other abnormal features have been found.

Effect of Ultraviolet Light Accompanying Cathode Rays—It is well known that air and all gases emit visible and ultraviolet light when bombarded by electrons of a few volts. Coolidge (5) showed that when cathode rays strike the molecules of air surrounding the tube they produce a luminous cone of light. Only light below 3100 \AA ., however, could play any part in producing vitamin D.

Qualitative tests showed the presence of ultraviolet light. For example, photographic paper was placed under a Wood's filter of glass, under quartz, under Pyrex, and also under ordinary glass. In each case the filter was covered with water to absorb the cathode rays. Upon operation of the tube, the blackening produced depended on the ultraviolet light transmission of the various filters. An image of the luminous cone focused with a quartz lens onto an anthracene plate showed fluorescence.

Quantitative tests were made with a spectrogram of the luminous cone. Analysis of the areas under the different lines procured with a recording microphotometer showed that of the total blackening of the plate 12 per cent was caused by light in the region from 3080 to 2800 \AA .

The earlier experiments of Knudson and Moore (4) in activating ergosterol under a quartz dish were repeated in order to check the evidence as to the relative importance of the cathode rays and the accompanying ultraviolet light in vitamin D synthesis. As will be seen later, however, in these experiments there was introduced the new variable of quartz fluorescence. The ergosterol was placed in the bottom of a quartz dish, inverted, and sealed tight

with paraffin to prevent any scattered electrons from gaining access to the sterol. The results of these experiments are presented in Table II.

The potency of 100 rat units per mg. obtained in 400 to 1000 seconds is better than any ever obtained in these experiments with the direct action of cathode rays on the unshielded ergosterol. Since the action here is produced entirely by ultraviolet rays of extremely low intensity, except for a very slight effect due to x-rays, it is reasonable to expect that, if it were practical to increase the exposure time still more, potencies of 1000 rat units per mg. or better would be reached, comparable with those obtained by the direct action of ultraviolet light. The abrupt increase in

TABLE II
Activation of Ergosterol under a Quartz Dish

Time	Weight	Potency	Total rat units
<i>sec.</i>	<i>mg.</i>	<i>rat units per mg.</i>	
30	24	<0.16	<4
60	24	<0.16	<4
120	24	<0.16	<4
240	24	<0.16	<4
300	5	10*	50
400	24	2.5*	60
400	5	100*-20	500-100
1000	5	100	500

* Potencies are estimated as explained in Table I.

potency after about 400 seconds explains why it was difficult to obtain checks with the experiments at 400 seconds which at first sight are in wide disagreement. Furthermore, it shows that if Knudson and Moore (4) had increased the time of exposure enough to reach this sharp threshold, they would have obtained activation behind a quartz plate. A similar "induction period" was found also with direct bombardment by cathode rays and it will be discussed later.

A study was next made of the fluorescence of the quartz itself. A spectrogram showed that ultraviolet light was produced by the action of cathode rays on quartz, but this emission was easily prevented by covering the quartz with a layer of water. The

results of experiments in which the fluorescence of quartz was eliminated are shown in Table III.

In Table III, Experiment B was performed with an ether solution in a quartz tube; in the others ergosterol was used in the solid state. In Experiment A the quartz dish used previously (Table II) was covered with water. In neither case was the area exposed the same as in the direct bombardment already described in Table I. Therefore, in Experiments C and D a special vessel was constructed, having a quartz window covered with water, below which was placed an 8 cm. nickel dish, the same as was used in the direct exposure to cathode rays. A lead shield at the bottom and sides excluded all stray cathode rays.

In Table I it is seen that an exposure of 2000 seconds to the direct action of the cathode rays produced 240 rat units of anti-

TABLE III
Activation of Ergosterol under Quartz and Water

Experiment	Time	Weight	Distance	Potency	Total rat units
	sec.	mg.	cm.	rat units per mg.	
A	100	24	6.0	0.2	4.8
B	900	15	8.8	0.14	1.2
C	1000	40	2.5	0.12	4.8
D	2000	44	2.5	0.12	5.3

rachitic material, whereas, according to Experiment D, under the same conditions, the ultraviolet light accompanying the cathode rays produced only 5.3 rat units or 2.2 per cent as much. Again, in Experiment C, for an exposure of 1000 seconds the ultraviolet light produced less than 4.8 rat units, whereas the full exposure to the cathode rays produced 408 rat units—less than 1.2 per cent.

Attempts were next made to eliminate the ultraviolet light entirely. Most of the effect of the air luminosity was excluded by placing a very thin sheet of aluminum foil directly over the layer of ergosterol. The foil transmitted the cathode rays but not the ultraviolet light. A potency of 10 rat units per mg. was reached in 400 seconds under these conditions. Experiments in a vacuum cell, already described, showed that antirachitic properties can be produced under these special conditions in the absence of air and of

luminescence from it; *i.e.*, by cathode rays alone. It is not certain, however, that the antirachitic product is identical with that produced by ultraviolet light.

Effect of x-Rays—In the operation of a cathode ray tube x-rays are produced. Tests with metal filters showed that in these experiments their effects are small, producing potencies of 0.07 to 1 rat unit per mg. in 400 seconds.

DISCUSSION

The effect of continuing exposure of ergosterol to cathode rays is shown in Table I. It is similar to that produced by continuing exposure to ultraviolet light, as found by several investigators. A maximum potency of 20 rat units per mg. is reached in about 400 seconds, after which the potency falls off. This is due, of course, to the simultaneous formation and decomposition of vitamin D by the same radiation.

A peculiar condition exists during the first 30 seconds of exposure. Apparently an early maximum or steady state occurs, as shown in Table I by the fact that the potency does not increase from a 5 second exposure to a 30 second exposure. An increase of practically 6-fold in vitamin D content would be expected.

Three reaction rates are of importance here: first, a gross rate of formation, decreasing from the start, as ergosterol is used up; second, a rate of vitamin decomposition which starts at 0 but increases as the amount of vitamin builds up; third, a net rate of vitamin accumulation, which is the difference between the rate of formation and the rate of decomposition. It is important to differentiate between formation and accumulation.

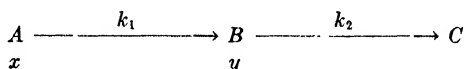
The tangent to the time-activation curve, which can be plotted from the data of Table I, represents this net rate of vitamin accumulation, and it is horizontal at both maxima. Since the net rate is 0, the gross rate of formation and the rate of decomposition at these points must be equal. The ratio of the amounts of vitamin present at the first and second maxima is roughly 0.2 to 20, or 1 to 100. The rate of decomposition must have increased 100-fold, since the decomposition rate is proportional to the amount present. Therefore, the rate of vitamin D formation is also 100 times greater at the second maximum, since a steady state obtains at these points. This increase is quite unexpected, because the rate of vitamin formation must decrease as ergosterol is consumed.

One possible explanation is a change of ergosterol into an isomer which has a much higher efficiency for conversion into vitamin D. This effect seems to fit in with the other peculiar phenomena occurring in the initial stages, such as the anomalous increase in the absorption coefficient for ultraviolet light, and the time lag involved in the exposure to weak ultraviolet light, as shown in Table II, in the experiments with ergosterol under quartz. This result is probably related to the formation of intermediate products (7). The initial formation of a product such as lumisterol might explain these phenomena.

Aside from this early anomaly, the time-activation curve as a whole can be reproduced satisfactorily by a kinetic treatment.

The maximum potency was 20 rat units per mg. If one takes the potency of 100 per cent vitamin D as 10 million rat units per gm., then at our maximum only 0.2 per cent of the ergosterol was converted into vitamin D. This low value (greatly lower than that attainable with ultraviolet light) suggests that the rate constant k_2 for the decomposition is greater than k_1 for the formation of vitamin D.

According to the simplest hypothesis, vitamin D may be assumed to accumulate as the result of two consecutive first order reactions as below.



Here A , B , and C represent ergosterol, vitamin D, and decomposition products respectively. At any time the amounts of the first two are designated by x and y .

At the maximum, the following equations apply. The maximum possible vitamin D content of ergosterol is represented by c_0 .

$$t_{\max.} = 1/(k_1 - k_2) \times \ln k_1/k_2 \quad (1)$$

$$y/c_0 = e^{-k_2 t} = k_1/k_2 \times e^{-k_1 t} \quad (2)$$

When k_1 is much smaller than k_2 , Equation 1 reduces to

$$-k_2 t = \ln k_1/k_2 \quad (3)$$

When Equation 2 is solved

$$0.002 = e^{-k_2 t}$$

$$k_2 t = 6.2$$

Substituting in Equation 3 we obtain

$$-6.2 = \ln k_1/k_2$$

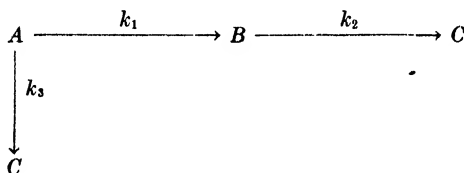
$$\log k_2/k_1 = 6.2/2.3 = 2.7$$

$$k_2/k_1 = 500$$

$$\text{Also } \log x/c_0 = -k_1 t/2.3 = (3.1 \times 10^{-5} \times 400)/2.3 = -0.0059$$

$$x/c_0 = 0.988 = 98.8\%$$

In order to obtain a steady state, when the vitamin D is present only to 0.2 per cent, the velocity constant for the decomposition must be 500 times greater than that for the formation. According to the calculations based on the above hypotheses, there should remain at the maximum more than 98.8 per cent of unchanged ergosterol. Obviously, such is not the case, for the product here is a yellow oil whose absorption spectrum is no longer characteristic of ergosterol. Clearly, the explanation is that either ergosterol or an intermediate such as lumisterol is largely decomposed directly by cathode rays without going through the vitamin D stage. A more complete hypothesis then comprises the following steps.



Here A , B , and C represent as before the ergosterol, vitamin D, and the decomposition products. This representation embraces the possibility of intermediate steps between A and B , since k_1 will refer to the slowest, or rate-governing step, if such isomers do enter. The following equation represents the amount y of vitamin D present at any time.

$$y = c_0 k_1 / (k_2 - k_1 - k_3) (e^{-(k_1 + k_3)t} - e^{-k_2 t})$$

Successive approximations give the constants in the above equation the following values.

k_1	k_2	k_3
0.0000174 sec. ⁻¹	0.0005 sec. ⁻¹	0.007 sec. ⁻¹

These enable one to calculate the time-activation curve mathematically as shown in the last column in Table I.

The agreement is satisfactory except near the beginning where the anomaly, already discussed, occurs. The implication of these constants is striking. It shows that the probability for the destruction of ergosterol is 400 times greater, and for the destruction of vitamin D 30 times greater than the probability of the formation of vitamin D from ergosterol by electrons at the velocity and under the conditions described here. This situation is plausible in view of the fact that the formation of vitamin D is a highly specific reaction requiring, according to Windaus and Thiele (8), the rupture of a ring with the production of an additional double bond, while on the other hand the decomposition need follow no definite, specific route. Further, these high speed electrons, because of their enormous energy, have lost much of the precision as a kinetic tool that characterizes selectively absorbed light quanta of smaller energy.

The actual mechanism of the production of vitamin D by cathode rays is an interesting problem. We have been unable to detect the presence of any secondary ultraviolet fluorescence from the bombarded ergosterol. The possibility is far from remote, however. The emission of x-rays or light upon the deceleration of electrons is quite general.

The output of the cathode ray tube measured calorimetrically is nearly 200 calories per minute. Calculations based on this output show that about 45×10^{16} electrons are emitted in 400 seconds. This figure is probably too high, because it is necessary to estimate the average voltage of the electrons by assuming that the wave form of the rectified potential is still a true sine curve. (With Thaller's (9) data for the electronic output of a tube, a value of 10×10^{16} electrons in 400 seconds is obtained.)

It is improbable that there is any fundamental integral relationship between the number of electrons absorbed and the number of vitamin D molecules produced because of the complex nature of the process. However, the following calculations are helpful in giving further understanding of the action of the cathode rays.

The number of ergosterol molecules originally present in the 24 mg. is 3.8×10^{19} . The number of vitamin D molecules at the maximum potency is 0.2 per cent of this, or 7.6×10^{16} . At the

maximum, $k_1x = k_2y$, therefore, $x = 0.002 \times 0.005/0.0000174 = 0.058$, or 5.8 per cent of unchanged ergosterol. Therefore, $3.8 \times 10^{19} \times (1 - 0.058) = 3.6 \times 10^{19}$ molecules have been changed.

The gross electronic yields as molecules per electron, M/E , are

$$M/E \text{ for antirachitic material found} = \frac{7.6 \times 10^{18}}{45 \times 10^{18}} = 0.2 \text{ molecule per electron}$$

$$\text{“ “ ergosterol decomposed} = \frac{3.6 \times 10^{19}}{45 \times 10^{18}} = 80 \text{ molecules per electron}$$

The average voltage per electron has been estimated at 77,000. This, of course, does not necessarily mean that each of the 80 molecules struck actually absorbed about 1000 volts of the electron's energy. Many collisions with the decomposition products for example would dissipate energy and such collisions are not accounted for. These figures do support the view, however, that in many cases the molecules may absorb much more than the 4 electron-volts (calculated as the equivalent of 3000 Å.) necessary for vitamin D formation by ultraviolet light and that much of the absorbed energy is consumed in the decomposition of the molecules or the conversion into heat.

The problem of activation by electronic bombardment will probably be attacked better by the use of slower electrons and by further knowledge concerning the action of cathode rays on simpler molecules.

The authors are greatly indebted to Professor Harry Steenbock and Dr. R. W. Haman for advice during the course of this investigation and to Dr. Henry T. Scott; and they are glad to acknowledge the help given by the Wisconsin Alumni Research Foundation.

SUMMARY

1. In agreement with the findings of earlier workers, ergosterol was found to acquire antirachitic properties when bombarded with high voltage cathode rays. Experiments showed that under these conditions the effect due to the accompanying x-rays and ultraviolet light is relatively small. Experiments were tried with various filters and in the presence and absence of air.

2. The cathode rays were found to exert a strong destructive

effect and the potencies were found to be considerably less than those obtainable with ultraviolet light.

3. Anomalous effects in the early stages of activation lend support to the hypothesis that ergosterol changes to an isomer preceding vitamin formation.

4. By a kinetic treatment the time-activation curve is shown to be governed by three simultaneous reactions, the formation of vitamin D, the decomposition of vitamin D, and the direct decomposition of ergosterol.

5. One high velocity electron produces, under the conditions described, less than 1 molecule of vitamin D but decomposes about 80 ergosterol molecules.

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METHOD FOR THE DETERMINATION OF THE NON- PROTEIN NITROGEN OF TISSUE

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The determination of non-protein nitrogen of tissue and the establishment of the nitrogen partition are of much help in the study of different problems of general physiology and biochemistry, as was shown recently, among others, by Terroine and Szucs (6), Bonnet (1), Roche (5), and Mezincesco *et al.* (4). Unfortunately the methods recommended for the extraction and determination of the soluble nitrogen are not very reliable. Terroine and Szucs, Bonnet, and Mezincesco and coworkers have employed the method of Voit (7) and Roche the method of Janney (3). With that of Elias and Kaunitz (2), these methods are practically the only ones actually employed. We will point out briefly their disadvantages.

The method of Voit was intended for food analysis, and its author used dry powdered substances as analytical material. When applied to the determination of non-protein nitrogen of fresh animal tissue, it very often gives aberrant results, because the alcoholic concentration of the extraction fluid varies with the magnitude of the sample and its water content, and also because the fluid is deeply colored and it is very difficult to control its pH. Besides, the analysis takes a long time and necessitates involved manipulations: grinding the sample with sand in a mortar, quantitative transference of the suspension into a volumetric flask, and distillation of the alcohol.

The method of Janney, although involving also long and tedious steps (four water extractions, four filtrations, 18 hours of alcoholic extraction), is simple enough and gives fairly close results. But, as was pointed out by the author, it gives far too high figures for the non-protein nitrogen, since part of the protein is not precipi-

tated. Compared to the total protein nitrogen, the quantities remaining in solution are very small and the method allows the determination of protein with a close approximation; but these quantities are considerable in comparison with the extractive nitrogen, the figures for the latter ranging from 10 to 15 per cent higher than the true ones.

Taking into account these facts, we thought that a simpler and more accurate method would be useful.

It appeared to us evident that such a method should be based on the use of one of the protein precipitants employed in blood analysis, trichloroacetic acid for instance, which has been thoroughly studied and recognized as a much better precipitant than alcohol and alcoholic and saline solutions. But the problem we had to deal with did not consist chiefly in the choice of a protein precipitant; indeed, the principal difficulty in the determination of the non-protein nitrogen of tissue is its complete extraction. Numerous experiments showed that grinding the tissue in a mortar with water or trichloroacetic acid solutions does not give good results. Most of the tissues cannot be ground well, even if they have been first passed through a meat grinder, and the protein-free extracts obtained in parallel analyses contain variable quantities of nitrogen (differences between 10 to 15 per cent in the analysis of beef muscle). At any rate it is to be noted that by grinding with water and subsequent addition of acid, more regular results are obtained. This shows that the protein coagulation prevents the diffusion of the soluble nitrogen, at least when the grinding is not adequate. Elias and Kaunitz obtain better extractions by freezing fine slices of the analytical material in carbon dioxide snow and grinding them with water and sand in a mortar. Our numerous experiments finally led to the following technique, which avoids freezing of the sample as well as grinding it in a mortar.

Method

About 100 gm. of tissue are passed through a small meat grinder. We use a medium size Latapie grinder, which gives a very fine hash, but any kind of meat grinder can be employed. Only, in comparison with the size of the instrument, the tissue quantity must be fairly large, since some remains in the grinder after its juice has

been pressed out, so that the composition of the ground material is not quite identical with that of the original.

The ground tissue is thoroughly mixed (if necessary with addition of some water) until it is transformed into a more or less homogeneous paste, and its dry weight determined by heating a sample at 105°. 3 to 7 gm. of the paste are weighed in a glass cylinder, with heavy walls and a glass stopper, of about 120 cc. capacity. Ten to twenty glass pearls about 8 mm. in diameter and 50 cc. of water are introduced into the cylinder; its contents are shaken vigorously for 10 minutes and then allowed to stand for 30 minutes. After this time, 50 cc. of a 20 per cent trichloroacetic acid solution are added, the cylinder is shaken again for 10 minutes, left for 3 hours in the refrigerator, and then its contents filtered. The filtrate must be perfectly clear. A good filter paper should be used and the first portions refiltered if necessary. The nitrogen is determined on part of the filtrate by the Kjeldahl method, and the result is referred to the total liquid volume (100 cc. plus water of the sample) and finally to the dry weight of the sample.¹

With very small amounts of tissue the meat grinder cannot be used and the sample is hashed with scissors. We employ curved dissecting scissors with fine blades. The tissue sample is weighed on a small watch-glass, thoroughly hashed, and then transferred quantitatively with 50 cc. of water into the glass cylinder. The procedure is continued as above.

EXPERIMENTAL

In Tables I to VI are given the results of some of the experiments made in order to verify the proposed method.

The data contained in Tables I and II show that for quantities of different tissues (beef muscle, pig liver, kidney, brain, etc.) below 10 gm. (fresh material), the extraction of the non-protein nitrogen is completed in 3 hours. They show also that, under the recommended conditions, the extraction of greater quantities gives too low figures; so, for the last sample (Table I)

¹ If water is added to the ground tissue and we want to determine the non-protein nitrogen concentration in the fresh material, the water content of the latter is determined and its non-protein nitrogen is calculated from the figure corresponding to the dry weight.

in the extraction of 20 and 23 gm., an average of 0.290 per cent of soluble nitrogen is obtained, against 0.310 when only about 10 gm. are extracted.

The results in Table III justify the method of calculation adopted. The figures show that we must take into account the

TABLE I
Influence of Quantity of Tissue

Sample	Quantity extracted	Non-protein N*
	<i>gm.</i>	<i>per cent</i>
Pig liver	6.6461	0.257
	10.2656	0.257
Sheep brain	6.2527	0.203
	10.8502	0.199
Pig kidney	4.8946	0.234
	9.7922	0.229
Beef Muscle I	2.9741	0.379
	5.3968	0.386
" liver	3.0707	0.248
	5.1188	0.242
" Muscle II	9.1648	0.305
	11.0364	0.315
	20.4730	0.289
	23.4943	0.291

* All the figures for the non-protein N concentration correspond to the fresh weight of the analyzed tissues.

TABLE II
Influence of Extraction Time

N extracted from fresh beef muscle in		
3 hrs.	8 hrs.	24 hrs.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.4078	0.4133	0.4078
0.4110	0.4177	0.4101

increase in the volume of the extraction fluid with that of the tissue water, and consider the volume of the nitrogen solution as equal to the sum, volume of trichloroacetic acid solution + volume of added water + volume of tissue water. Indeed, if the extraction fluid volume is considered as equal to 100 cc., the results are too low; and the greater the sample the lower the results,

The figures given in Table IV show that reliable enough results can be obtained without passing the sample through a meat grinder. Nevertheless, to obtain correct figures without grinding is sometimes difficult and a meat grinder should be used whenever sufficient tissue is available.

The degree of accuracy of the method is shown by the figures in Table V. They prove that differences between parallel analyses rarely exceed 3 per cent. „The least favorable results were ob-

TABLE III
Verification of Calculation

Sample	Quantity extracted	Corresponding amount of water	Soluble N when volume of solution is considered	
			100 cc.	100 cc. + tissue water
	<i>gm.</i>	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>
Pig liver	6.6461	5.26	0.244	0.257
	10.2656	8.12	0.238	0.257
Sheep brain	6.2526	5.16	0.194	0.203
	10.8502	8.96	0.182	0.199

TABLE IV
Data Obtained without Grinding

Sample	N extracted	
	With grinding	Without grinding
	<i>per cent</i>	<i>per cent</i>
Beef muscle	0.439	0.437
	0.444	
Pig liver	0.378	0.381
	0.386	0.380

tained with beef muscle. It is noteworthy, moreover, that butchers' meat gives less regular results than fresh tissues.

In Table VI we give comparative figures obtained by the method of Janney and by trichloroacetic acid extraction in parallel analyses of two meat samples. As predicted, the method of Janney leads to soluble nitrogen concentrations about 10 per cent higher than those obtained by our method. The differences correspond to the protein not precipitated by boiling water.

TABLE V
Results of Parallel Analyses of Different Tissues

Sample	Quantity extracted	Non-protein N	Average	Maximum difference, per cent of average
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>	
Pig liver	2.3989	0.273	0.271	2.58
	3.5837	0.266		
	3.7744	0.273		
	3.0733	0.271		
Calf Muscle I	5.1188	0.242	0.246	2.44
	4.3814	0.248		
	4.3520	0.247		
	3.0707	0.248		
" " II	3.4570	0.243	0.279	1.41
	5.7931	0.280		
	6.4707	0.280		
	5.3607	0.276		
Rabbit muscle	4.0323	0.279	0.443	2.71
	4.0270	0.437		
	4.5036	0.444		
	3.9555	0.439		
Pig kidney	4.1649	0.449	0.230	2.61
	4.3653	0.444		
	7.5434	0.231		
	9.7922	0.229		
Beef Muscle I	7.7994	0.231	0.314	3.50
	9.2702	0.225		
	4.8946	0.234		
	9.0476	0.320		
Sheep brain	8.5673	0.309	0.200	1.50
	7.1402	0.315		
	8.4657	0.317		
	6.8137	0.309		
Beef Muscle II	6.2527	0.203	0.363	3.30
	7.6317	0.198		
	10.8502	0.199		
	6.5543	0.201		
	8.4861	0.359		
	6.1672	0.371		
	7.6679	0.362		
	6.6180	0.362		

In consideration of the fact that it is almost impossible to establish a satisfactory direct verification of the methods for determining tissue non-protein nitrogen, we consider the above figures conclusive enough. They show that the method gives coherent and regular results. Under the conditions recommended, no correlation between the magnitude of the sample or the extraction time and the quantity of nitrogen extracted can be ascertained; this indicates that the extraction is complete. On the other hand, the quantities of nitrogen dissolved in 3 hours by protein hydrolysis cannot be significant. Thus we can consider the figures we obtain as corresponding to the true non-protein nitrogen con-

TABLE VI

Comparison between Method of Janney and Trichloroacetic Acid Extraction

Sample	Soluble N found by	
	Trichloroacetic acid extraction	Method of Janney
	<i>per cent</i>	<i>per cent</i>
Beef Muscle I	0.406	0.444
	0.404	0.438
Average.....	0.405	0.441
" difference.....	8.89	
Beef Muscle II	0.374	0.416
	0.373	0.413
Average.....	0.3735	0.4145
" difference.....	10.98	

centration. To be sure, by this method the protein nitrogen can be determined by difference, and the trichloroacetic acid extract can be employed for the determination of the different soluble constituents.

SUMMARY

A method for the determination of the non-protein nitrogen in tissue, based on trichloroacetic acid extraction, is described, and data obtained by this method are discussed.

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DETERMINATION OF THE NITROGEN PARTITION IN TISSUES*

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In some investigations on intermediary protein metabolism it has been found necessary to devise and adapt methods for determining certain protein and non-protein nitrogenous constituents of various tissues and of the carcass as a whole. A number of workers (1) have contributed methods for the determination of one or several tissue constituents, but so far as we are aware no such system as that of Folin and Wu (2), as is now widely used for blood analysis, has been developed for tissues. Borsook (3) has recently developed spectrophotometric methods which may be adapted for tissues.

The main objects sought for this system were, (a) the use of tissue filtrates containing the constituents to be determined in convenient concentrations for colorimetric analysis, without interfering chemical reagents; (b) a technique of preparation of the tissue extracts which would prevent destruction or change of the constituents either by autolytic enzymes or by the reagents used.

By the use of two tissue filtrates, the methods described below permit the determination of the following constituents: (a) soluble proteins, albumins, and globulins in a buffered salt extract; (b) sugar and soluble non-protein nitrogenous constituents, including amino acids, ammonia, urea, creatine, creatinine, allantoin, glutathione (reduced), uric acid, and non-protein nitrogen in a tungstic acid extract. In addition certain other determinations such as glycogen, calcium, phosphorus, sulfur, total nitrogen, fat, ash,

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cystine, cysteine, methionine, and water may be made on other samples of the tissue.

Methods

Adult rats of the Wistar strain were used as the experimental animals. In studies on the nitrogen partition of tissues it is especially important that careful attention be given to the nutritive condition and the selection of the animals used. The paired feeding method of Mitchell and Beadles (4) has been found to be particularly suitable in the control of nutritional factors.

The rats, after a known period of fasting, are killed by a blow on the head. The abdomen is quickly opened and the bladder and its contents clamped off and removed. The liver or other special tissue desired is next removed and dropped into a small pan half full of cracked CO₂ ice. It is manipulated by one operator so that it is quickly frozen solidly. Meanwhile the whole alimentary tract within the abdomen is removed, care being taken to leave behind most of the mesentery and the pancreas. The carcass minus the bladder, liver, and alimentary tract is then quickly chopped into several pieces and put through an electric (1 horse power) meat grinder having a cutting plate with holes either $\frac{1}{8}$ inch or $\frac{5}{16}$ inch. The grinder should be cooled with powdered CO₂ snow and the hashed tissue collected in a pan containing granular CO₂ snow. The tissue is thus quickly reduced to a low temperature. Usually from two to six rats are combined for any one experiment in order to obtain enough liver tissue for all of the determinations and to average individual variations in the animals. The cold hashed tissue is put through the grinder several times to insure thorough mixing and finer hashing. It is then spread over large chunks of CO₂ ice, covered, and kept frozen until samples can be weighed.

The frozen livers, together with pieces of CO₂ snow, are put several times through a chilled hand grinder with a crushing end. The liver tissue comes out well mixed in a dust-like powder. This powder is kept frozen until samples are taken. The hashed and frozen whole carcass can likewise be reduced nearly to a powder in the same way, but this is unnecessary for most purposes. The technique of Gracser, Ginsberg, and Friedemann (5), by which liquid air is used for freezing, has been tried, but for the present

determinations the procedure given above, with CO_2 snow, has proved more satisfactory. The inert and acid qualities of CO_2 are protective to such constituents as glutathione. The snow is cheap and easily procurable and the hashing is comparatively easy. In humid weather moisture from the air may condense on the frozen tissue unless it is kept covered.

Tissue Filtrate for Non-Protein Nitrogenous Constituents

From 10 to 50 gm. of the frozen hashed or powdered tissue are weighed into 8 volumes of cold $\text{N}/12 \text{ H}_2\text{SO}_4$ and allowed to stand with occasional shaking for 2 or 3 minutes. 1 volume of cold 10 per cent sodium tungstate and 2 to 10 gm. of washed and ignited sand are added. The mixture is shaken vigorously for 10 to 30 minutes. The extract is filtered through a fine pore quantitative filter paper and kept in the ice box until used. The filtrate should be just acid to methyl red. A few drops of toluene are added as a preservative. If sugar is to be determined, toluene must not be added.

Other precipitants than tungstic acid were tried. Sulfosalicylic acid (4 volumes of 10 to 20 per cent) extracts are difficult to filter. Trichloroacetic acid (4 volumes of 10 to 15 per cent) is satisfactory except in three respects. Much calcium phosphate dissolves in the strongly acid solution, causing troublesome precipitates on neutralization. A slow conversion of creatine to creatinine occurs in the strong acid, even in the ice box. It is impossible to recover uric acid quantitatively with this acid. Schaffer and Lee (6) analyzed tissues for several nitrogenous constituents, using 4 volumes of 3 per cent trichloroacetic acid.

The non-protein constituents are relatively stable in the tungstic acid filtrate and if kept at low temperature remain unchanged for several days. As far as we have been able to determine all of the non-protein nitrogen constituents are easily soluble in either tungstic or trichloroacetic acid in the dilutions used.

Determination of Non-Protein Nitrogen—The non-protein nitrogen is determined by the Koch-McMeekin (7) micro-Kjeldahl procedure. The original Koch-McMeekin digestion mixture was modified by using a 1:1 mixture of H_2SO_4 and saturated K_2SO_4 solution, containing 0.5 per cent CuSO_4 .

It is advisable to use gum ghatti in all nesslerizations. For

whole carcass a 1 cc. sample of the filtrate is used; for liver, 2 cc.; and for muscle, 1 cc. A 0.2 to 0.3 mg. standard is used.

Ammonia—Ammonia is determined by aeration, 20 cc. of the filtrate and 2 drops of capryl alcohol being used to prevent foaming. The technique used is that of Folin (8) for blood, except that the amounts of the sample and of the carbonate-oxalate solution are doubled. A 0.05 mg. standard is used.

Amino Acids—2 cc. of filtrate are diluted with 7 cc. of water and 1 cc. of 0.07 N HCl and the amino acids are determined according to Danielson's (9) procedure. A 0.1 mg. of *l*-leucine standard is used. In whole carcass extracts traces of calcium phosphate occasionally precipitate on standing. These may be removed by centrifuging.

Creatine—2 cc. of whole carcass or muscle filtrate are diluted to 25 cc. 5 cc. of this diluted filtrate are placed in a 30 cc. test-tube graduated at 10 cc. 1 cc. of N HCl is added, the tube is capped with tin-foil and heated in a boiling water bath for 3 hours or autoclaved at 120° for 1 hour. It is then cooled, the mixture is neutralized with 1 cc. of N NaOH and diluted to 10 cc. with water and 5 cc. of alkaline picrate solution are added (1 cc. of 10 per cent NaOH to each 5 cc. of saturated picric acid solution). A standard of 20 cc. of 0.01 mg. of creatinine per cc. is used. 10 cc. of alkaline picrate are added. The contents of the tubes are mixed and allowed to stand 15 minutes before reading. In computation note that the volume of the standard is twice that of the unknown. The pre-formed creatinine is subtracted from the creatine value. Since there is very little creatine or creatinine in the liver, these determinations were not made on liver filtrates.

Creatinine—For whole carcass or muscle extract, 10 cc. of filtrate are taken and 5 cc. of the alkaline picrate solution, made as above, are added. A 0.20 mg. standard is used (see above). Some calcium phosphate in whole carcass extracts may precipitate in this test. It is removed by centrifuging.

Reduced Glutathione—Glutathione is determined after the method of Benedict and Gottschall (10), except that the amounts of sample and of reagents are doubled and a final dilution to 25 cc. is made with 2 per cent sodium bisulfite. 10 cc. of muscle or whole carcass filtrate or 2 cc. of liver filtrate + 8 cc. of water are used. A 0.2 to 0.5 mg. of glutathione standard is used.

Urea—To 5 cc. of filtrate 2 drops of Folin's (8) urease buffer and

a strip of urease paper are added. Digestion is carried on for 30 minutes at room temperature. The ammonia formed is determined by aeration as in the blood method. The preformed ammonia is subtracted to correct the urea value.

Uric Acid—A modification of the method of Morris and Macleod (11) is used. To 8 cc. of whole carcass or muscle filtrate, or to 4 cc. of liver filtrate + 4 cc. of water, 0.2 cc. of 2.5 per cent ZnCl_2 and 1.6 cc. of 10 per cent Na_2CO_3 are added with mixing. After centrifuging, the supernatant fluid is discarded. The precipitate is dissolved in 10 cc. of Folin's (8) cyanide-urea reagent and the uric acid determined by Folin's method. A 0.004 to 0.008 mg. standard in 4 cc. of water is treated similarly to the tissue filtrate.

Allantoin—A preliminary purification of the filtrate is carried out partly according to Larson's method (12). It is not necessary to use phosphotungstic acid with these filtrates. 10 cc. of the regular tungstic acid tissue filtrate are placed in a 15 cc. centrifuge tube and 2 cc. of basic lead acetate solution are added. After centrifuging, the supernatant liquid is transferred to another 15 cc. tube, the lead precipitated with H_2S , and the PbS removed by centrifuging again. The supernatant solution is transferred to another tube and the H_2S removed by aeration. 10 cc. of the aerated solution are transferred to a 15 cc. graduated centrifuge tube and neutralized to phenolphthalein with N NaOH . 1 cc. of mercuric acetate reagent (according to the method of Read and Chaikoff (13)) is added. After standing for 30 minutes the mixture is centrifuged and the supernatant liquid discarded. The precipitate is dissolved with 5 cc. of 0.1 N HCl , the mercury precipitated with H_2S , and the HgS removed by centrifuging. The supernatant liquid is transferred to an ammonia aeration tube, the H_2S removed by aeration, and the allantoin hydrolyzed to urea by the method of Allen and Cerecedo (14). By this method the solution is neutralized, made to approximately 0.2 N with 3 cc. of N KOH , and heated at 70° for 2 hours in a water bath. The solution is neutralized, made to 0.1 N with N HCl , and heated to 70° for 30 minutes. The solution is again neutralized and the urea determined by the method described above. A 0.01 mg. standard is used. The nitrogen value is multiplied by 2.824 and by 1.2 to give allantoin and to correct for volume changes.

Sugar—Glucose is determined by the difference in the amounts

of reducing substances in tissue filtrates before and after treatment with washed yeast. The total reducing substances are determined on 1 cc. of filtrate according to Folin's (8) macromethod for glucose with a 0.2 mg. standard.

The glucose is removed quantitatively by yeast, and washed according to Somogyi's (15) method. A weighed amount of brewers' yeast is suspended in 5 to 10 parts of water. The mixture is centrifuged and the supernatant liquid discarded. This is repeated until a clear supernatant liquid, giving a negative test for reducing substances, is obtained. The yeast is suspended in 5 parts of water until ready for use. About five washings are necessary completely to free the yeast of reducing substances. The yeast suspension will keep for several days in the ice box, but it is necessary to wash the yeast once each day it is used. The glucose is removed quantitatively by a modification of Somogyi's (16) method.

5 cc. of the above yeast suspension are placed in a 15 cc. centrifuge tube, centrifuged, and the supernatant liquid discarded. 10 cc. of tissue filtrate are added and the mixture well stirred and incubated at 30° for 10 to 30 minutes. After centrifuging, 2 cc. of the supernatant liquid are taken for determination of the reducing substances as above, a 0.2 mg. glucose standard being used. The value is subtracted from the total amount of reducing substances present before treatment with yeast to get the amount of glucose.

The amount of non-glucose reducing substance varies, but in general whole carcass filtrates will give more than liver filtrates. An average value for whole carcass filtrates is 150 mg. per cent and for liver filtrates 120 mg. per cent.

Soluble Protein Constituents (Albumins and Globulins)

About 2 gm. of the frozen, powdered tissue are extracted with 50 volumes of 0.6 M KCl buffer (0.025 M K_2HPO_4 , 0.005 M KH_2PO_4) by gentle stirring for 2 hours. A few drops of toluene are added as a preservative. The mixture is filtered through a fine pore, quantitative filter paper and the filtrate is immediately ready for use.

The buffered KCl extraction solution employed is similar to that used by Edsall (17) for the extraction of muscle globulin and albumin, except that a considerably greater volume of half the

concentration is used. It was found that at least 25 volumes were required for complete extraction but that the concentration of KCl between 3 and 10 per cent was unimportant. Filtration was better with 50 volumes than with 25. Clear filtrates were always secured. The great dilution and probable destruction of certain non-protein nitrogen constituents, notably glutathione, precludes the use of this filtrate after protein precipitation for non-protein nitrogen determinations.

The protein extracted by 0.6 M KCl buffer solution is determined by the Koch-McMeekin (7) micro-Kjeldahl procedure, with a 1 cc. sample. The globulin is precipitated from a 20 cc. sample of the salt extract by dilution to 50 cc. with saturated Na_2SO_4 solution at 37° . It is allowed to stand for 3 hours at 37° , and then filtered. 3 cc. of the filtrate are taken for a micro-Kjeldahl digestion. This gives albumin nitrogen. Globulin nitrogen is considered to be the difference between soluble protein and albumin nitrogen. A correction for the non-protein nitrogen may be made.

Computations

It is necessary to allow for the moisture content of the tissues in computing results. The average water content of rat (adult) liver is about 72 per cent; muscle 70 per cent; and whole carcass 62 per cent. A variation of a few per cent in the moisture content does not alter the final results significantly.

The following formula shows how the results may be computed.

$$(R/X) \times S \times (V_1/V) \times (100/W) = \text{mg. per 100 gm. fresh tissue}$$

where R = colorimetric reading of standard
 X = " " " unknown
 S = value of standard in mg.
 V = volume of sample of filtrate for determination
 V_1 = " " tungstic acid solution used (volume of $\text{N}/12 \text{ H}_2\text{SO}_4$ + volume of 10% sodium tungstate) + amount of water in tissue sample
 W = weight in gm. of tissue sample

Several typical tissue analyses are given in Table I.

Recovery of Added Constituents

Two extracts of 50 gm. each of whole rat carcass were made according to the above technique. Before extraction leucine,

urea, creatine, glutathione, uric acid, allantoin, and glucose were added to one tissue sample. The results of analyses made on the two filtrates are given in Table II.

TABLE I
Typical Analyses of Tissues by Methods Described

Unless otherwise stated the results are given in mg. per 100 gm. of tissue.

Tissue	Amino acid N	Ammonia N	Creatine (as creatinine)	Creatinine	Glutathione (reduced)	Sugar	Urea	Uric acid	Non-protein N	Salt-soluble protein N	Albumin N	Globulin N	Moisture
													per cent
Whole carcass, normal adult ♀ rats.	45.0	5.0	175	9.5	38.0	40	21.0		245	1200	1000	200	62.0
Liver, adult ♀ rats.....	54.0	2.6			190.0	190	13.9		148	1600	1200	400	72.0
Liver, normal adult mice.....	58.4	6.2			260.0		19.0	3.9	222				74.1
Muscle, normal adult ♀ rats....	53.5	6.1	300	14.2	40.1		17.5	5.3	286	1420	1080	340	75.0

TABLE II
Recovery of Added Constituents

Unless otherwise stated the results are given in mg. per 100 gm. of tissue.

	Allantoin	Amino acid N	Ammonia and urea N	Total creatinine	Glutathione	Sugar	Uric acid	Non-protein N
Original filtrate	Trace	42.0	34.6	180	36.0	40	1.2	200
Amounts added	10	200	50	50	30	30	5	70
“ recovered.....	9.7	195	51	48	29	32	4.6	68
Percentage recovery.....	97	97.5	102	96	96.8	106.8	92	97.1

Other Determinations

Water, fat, ash, and total nitrogen are determined on convenient samples of fresh tissue by conventional methods. For total cystine, a 3 gm. sample of fat-free, dry tissue is hydrolyzed and the cystine determined by the method of Folin and Marenzi (18).

Cystine and cysteine may also be determined on a sample of fresh tissue treated with trichloroacetic acid according to the method of Mirsky and Anson (19).

For calcium, a 2 gm. sample of fresh tissue is digested with fuming nitric acid and the calcium determined by the permanganate method. Phosphorus is determined by Johnson's method (20) on a 2 gm. sample digested according to the method of Neumann (21). Sulfur is determined gravimetrically as BaSO_4 from a 10 gm. sample digested with fuming nitric acid and oxidized with bromine or H_2O_2 .

Glycogen is transformed into glucose by the Cori and Cori method (22) and the glucose determined by Folin's macromethod (8). Methionine is determined by Baernstein's method (23), with a 1 gm. sample of dry, fat-free tissue.

SUMMARY

A method of tissue analysis is described for determining soluble protein, albumins, and globulins; and the non-protein nitrogenous constituents, *viz.* amino acids, ammonia, creatine, creatinine, reduced glutathione, urea, uric acid, allantoin, and non-protein nitrogen. Two filtrates are used, a KCl-buffered extract for the soluble protein and a tungstic acid extract for the non-protein nitrogenous constituents.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

I. THE MEASUREMENT OF THE INHIBITION OF BLOOD CLOTTING. METHODS AND UNITS*

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Progress in the study of heparin and synthetic anticoagulants is conditional upon the availability of a simple method for the estimation of the activity of substances which inhibit blood clotting. Workers in this field have not as yet agreed upon one method, so that many of the results reported in the literature are subject to considerable uncertainty.

The method chiefly employed is that of Howell (1). According to this procedure 1 heparin unit is the amount of heparin which in the cold prevents 1 cc. of cat blood from clotting in 24 hours. This method has also been used by Charles and Scott (2) in their important work on the heparin content of various organs and on the purification of heparin. Fischer and Schmitz (3), and Schmitz (4), employing chicken plasma, use a "coagulation value" which is derived from a logarithmic function of the clotting times at 40° at various dilutions of heparin. Jorpes (5) in his work on heparin makes use of ox blood, the coagulation of which is determined at room temperature. From an inspection of the data on heparin activity before and after purification, given in the papers mentioned above, it will be apparent that the methods described can by no means form the basis of a comparative investigation of various anticoagulants.

We have tried to develop a simple method for the determination of inhibiting substances and to define an inhibitor unit which,

* Study of the mechanism of thrombosis and embolism supported by the Carnegie Corporation of New York.

within a certain limit of error, will be constant for a given substance.

EXPERIMENTAL

In the experiments here described chicken plasma was used. This, in contrast to that of almost all other animals, can easily be so secured that it will not clot for a relatively long period of time. On the other hand, by the addition of varying amounts of muscle extract any desired clotting time can be attained.

Examination of the conditions under which inhibitor activity could be estimated indicated that success depended on the following conditions. (1) The reaction volume must be kept constant, as the numerous substances contained in plasma respond to dilution in different ways. The addition to a series of plasma samples, for instance, of increasing amounts of an inhibitor solution will lead to discordant results. (2) Extreme care must be taken to disturb the plasma as little as possible. Attempts to increase the precision of the end-point may adversely affect its accuracy. Plasma which coagulates in the presence of an inhibitor in general gives rise to clots that are very soft and may easily be broken up beyond recognition, if shaken before their formation is complete. (3) The period of time over which the determination of inhibitor activity takes place should not be too extended, as the clotting properties of plasma may change quite considerably with time.

Method

Preparation of Plasma—The blood used in our experiments is obtained in the usual manner from the carotid artery of 2 year-old roosters, about 50 cc. being secured per animal. All glass parts with which the blood comes in contact are paraffined. The blood is collected in four fractions, the first and the last of which may clot faster. The chilled glass cups are immediately centrifuged in the angle centrifuge at 3000 R.P.M. for 20 minutes. The perfectly clear plasma is drawn off into paraffined tubes and kept in the refrigerator. In general, no plasma older than a week, nor any sample in which even a partial clot has formed, should be used.

Preparation of Activator—The activator is prepared according to the method described by Fischer (6) for his "muscle coagulin." From 165 gm. of breast muscle of a bled rooster 5.4 gm. of an

extremely active, slightly yellow powder were obtained. The addition of as little as 0.1 microgram in 0.03 cc. of physiological saline to 0.1 cc. of plasma effected a drop in clotting time from 120 to 90 minutes at 30°.

Definition of Unit—We define the inhibitor unit as the smallest amount of inhibitor which will raise the clotting time of 0.1 cc. of plasma to 4 times its normal value under the experimental conditions described in the next paragraph.

TABLE I
Estimation of Inhibitor Activity

Each tube contains 0.03 cc. of inhibitor solution, 0.1 cc. of chicken plasma, and 0.02 cc. of activator solution. Experiment I, 1.6 mg. per cc. (= 1:1) of Heparin R; Experiment II, 2.2 mg. per cc. (= 1:1) of Inhibitor C-L. + = clotted; ± = not completely clotted; — = not clotted.

Tube	Inhibitor dilution	Control		Experiment I			Experiment II		
		Clotting after							
		7 min.	8 min.	30 min.	60 min.	180 min.	30 min.	60 min.	180 min.
A		+							
B		—	+						
1	1:1			—	—	—	—	—	—
2	1:2			—	—	—	—	—	—
3	1:4			—	—	—	—	—	—
4	1:8			—	—	—	—	—	—
5	1:16			—	—	—	—	—	—
6	1:32			—	—	—	+		
7	1:64			±	+		+		
8	1:128			+					

Estimation: Experiment I, 1100 inhibitor units per mg.; Experiment II, 250 inhibitor units per mg.

Measurement of Inhibition—The measurements are carried out in a water thermostat at 30° ± 0.1°. A number of metal stands are suspended in the thermostat in such a manner that they can be freely turned over. These stands, which are similar in design to those described by Fischer (7), carry small Pyrex tubes (10 × 75 mm.) tightly closed by a cover that is screwed to the stands. In each experiment a fresh strip of waxed paper is placed between the rubber lining of the cover and the tubes.

A "normal clotting time" of between 6 and 10 minutes appeared to give the best results and was chosen in our experiments. In our experience, the addition of 0.02 cc. of an approximately 0.03 per cent solution of the activator to 0.1 cc. of plasma almost invariably led to a clotting time within this range. The actual concentration necessary has to be ascertained for each batch of plasma.

Each tube contains a glass bead of 4 mm. diameter, 0.03 cc. of the solution of the inhibitor at various dilutions in physiological saline, and 0.1 cc. of plasma. After all the tubes have been filled, 0.02 cc. of the activator solution is added, the tubes are closed, inverted once, and suspended in the thermostat. The first reading is carried out after a period corresponding to 4 times the normal clotting value, which has been determined in a preliminary experiment with 0.03 cc. of saline instead of the inhibitor. The complete immobility of the glass bead is taken as the criterion of clotting. As a check on the results the readings are repeated after 8 and 12 times the normal clotting time. It sometimes occurs in the first reading that at a certain inhibitor dilution the tube contents are viscous but not completely coagulated. If at this dilution the sample is found to be clotted in the subsequent reading, the inhibitor activity (expressed in inhibitor units per mg.) is taken as 20 per cent lower than actually calculated (see Table I, Experiment 1). All determinations are carried out in duplicate. Two typical experiments are given in Table I.

DISCUSSION

It is obvious that the method here described can only have the accuracy of a biological test and not that of a quantitative chemical procedure. The chief reason is the fact that the end-point, namely the formation of a clot, is comparatively ill defined. It nevertheless seems preferable to determine this end-point rather than some other property related to the clotting in an unknown manner, *e.g.* the transmission of light through plasma.

Attempts were made to increase the precision of the method by examining intermediate concentrations of the inhibitor. When in a given case the dilution 1:4 had been found active and the dilution 1:8 inactive, four intermediate dilutions were examined. Another method is to start from two solutions of the same in-

hibitor in which the relative concentrations are 2:3 and to compare the activities in both dilution series. Thus, an intermediate value can be found which in general will be more accurate than the one found in a single series. It has proved useful to establish an inhibitor standard with which to control the freshly obtained plasma preparations in order to be independent of individual variations. In these experiments a heparin preparation from beef lungs was chosen, the activity of which had repeatedly been found to be between 800 and 1000 inhibitor units. 1 inhibitor unit determined by the method described here appears to correspond roughly to 0.07 Howell unit. The activity of two heparin samples which are on the market was estimated. A heparin preparation of 15 Howell units per mg. obtained from the Connaught Laboratories, Toronto, Canada, was found to have 220 inhibitor units per mg.; another sample of 5 Howell units per mg., obtained from Hynson, Westcott and Dunning, Inc., Baltimore, contained about 70 inhibitor units per mg.

The accuracy of the method could be increased if it were possible to establish an inhibition curve. We have not been able, however, to determine such curves, contrary to the observations of Fischer and Schmitz (3). While it is perfectly easy to obtain a characteristic activation curve, the action of the inhibiting substance seems to have a different mechanism. A large number of experiments showed that there exists a minimum dose of the inhibitor which will keep the plasma liquid for many hours. Two typical experiments are given in Table I in which an inhibitor dilution of 1:64 and 1:32 respectively did not prevent clotting within 30 minutes, whereas the samples containing the next higher concentration stayed liquid for 3 hours and more. Whether this phenomenon is connected with the softening effect exerted by heparin on the fibrin clots, as mentioned before, or whether we are dealing here with a special "poisoning effect," we do not know. In the case of heparin the animal organism seems to be able to destroy its inhibiting properties in a comparatively short time. It may be different with some of the synthetic anticoagulants. It will be a matter of further work to determine whether this minimum dose coincides with the toxicity level of certain inhibitors.

We are indebted to Mrs. Charlotte Breitung for assistance in the course of these experiments.

SUMMARY

1. The conditions are discussed under which the inhibition effect of heparin and similar substances on plasma clotting can be measured.

2. A method is described for the estimation of inhibitor activity, and a definition is given of the inhibitor unit.

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STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

II. ON THE INHIBITION OF BLOOD CLOTting BY SUBSTANCES OF HIGH MOLECULAR WEIGHT*

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The discovery in liver of the anticoagulant heparin by Howell and Holt (1) probably marks the greatest progress in the theory of blood clotting since the times of Schmidt and Hammarsten. The fact that representatives of both classes of substances having antagonistic effects in blood clotting, *viz.* the activators and inhibitors, occur in animal tissue may form the basis for the assumption that there normally exists in the body an equilibrium between these substances, which is disturbed in certain pathological cases (*e.g.* thrombosis, hemophilia, etc.).

Howell (2) was the first to draw attention to the fact that heparin was probably formed of a complex containing uronic acids, calcium, and sulfuric acid. The importance of the $-\text{SO}_3\text{H}$ group for the inhibition of blood clotting was emphasized by Demole and Reinert (3) and by Fischer (4). The high ash content of heparin has been pointed out by almost all workers on this subject. Jorpes (5) has recently reported the isolation from heparin, prepared from ox and horse liver according to the method of Charles and Scott (6), of a substance which he considers to be a chondroitintrisulfuric acid. According to Schmitz (7), however, heparin prepared by the procedure of Schmitz and Fischer (8) does not contain sulfuric acid. While the work here described was in progress, Bergström (9) reported on a number of polysaccharide sulfuric acids with heparin activity.

* Study of the mechanism of thrombosis and embolism supported by the Carnegie Corporation of New York.

The findings summarized in the preceding paragraph suggest one of the following possibilities. (1) Heparin may actually be a polysaccharide sulfuric acid, in which case the results obtained by Schmitz (7) could not be explained. (2) The animal body may contain more than one "heparin." Although this assumption would account for the isolation of both sulfur-free and sulfur-containing substances, it lacks conclusive evidence. (3) The heparin preparations described in the literature may contain only small amounts of the active substance. In this case the inhibition of blood clotting by synthetic anticoagulants might be governed by a mechanism entirely distinct from that of heparin inhibition and could not serve as a model for this action. To test these

TABLE I
Substances Active in Inhibition of Blood Clotting

Experiment No.	Substance	Inhibitor units per mg.
1	Sodium salt of cellulose sulfuric acid (10, 11)	80
2	" " " " disulfuric "	250
3	Potassium salt of polyvinyl sulfuric acid	250
4	Sodium salt of galactan sulfuric acid from red alga, <i>Iridex laminarioides</i> (12)	30
5	Heparin H	70
6	" T	220
7	" R	1100

possibilities an investigation was carried out of the effect on blood clotting of a number of naturally occurring and synthetic substances of high molecular weight.

Our findings are summarized in Tables I and II. It will be seen that no substance active in the inhibition of blood clotting could be found that was free of sulfur. It should, however, be stated that a product obtained by phosphorylation of cellulose with phosphorus oxychloride showed weak inhibition activity (about 15 inhibitor units per mg.). As this substance has not as yet been purified to a satisfactory degree, we shall have to reserve this point for the future. On the other hand, a number of substances of high molecular weight, containing sulfuric acid esters, were found inactive (Table II, Experiments 5, 7, 9). The inactivity of

cellulose monosulfuric acid may be due to its low solubility in water. The lack of activity of agar-agar and the polysaccharide from cornea may be explained by the assumption that the type of sulfuric acid linkage in these substances is different from that in the inhibitor substances. The inactivity of pentamethylene and decamethylene disulfonic acids (Table II, Experiments 1 and 2) is in agreement with the findings of Bergström (9) regarding the sulfuric acid derivatives of simple sugars which likewise were found inactive.

TABLE II
Substances Inactive in Inhibition of Blood Clotting

Experiment No.	Substance	Concentration per cc. plasma
		<i>mg.</i>
1	Sodium salt of pentamethylene- α, ω -disulfonic acid (13)	7.4
2	Sodium salt of decamethylene- α, ω -disulfonic acid (13)	8.2
3	Sodium salt of taurocholic acid (synthetic) (14)	4.3
4	Polyvinyl alcohol	3.1
5	Sodium salt of cellulose monosulfuric acid	1.3
6	β -Amylose	2.7
7	Agar-agar	1.2
8	Polysaccharide from vitreous humor (15)	1.6
9	“ “ cornea (16)	2.2
10	Specific polysaccharide from pneumococci Type III (17)	1.2
11	Specific polysaccharide from bacillus Calmette-Guérin (18)	1.5
12	Peptone (Hoffmann-La Roche)	1.9
13	Taka-diastrase (Parke, Davis)	1.2
14	“Antitrypsin” from egg white (19)	1.9

The fact that the comparatively strong polysaccharide acid from Type III pneumococci, which gave highly viscous solutions in water, failed to show any inhibiting activity (Table II, Experiment 10) proves that high molecular weight and acidic properties are not the only requirements for a potent inhibitor. “Anti-trypsin” (19), a preparation from egg white inhibiting the action of trypsin, was inactive in blood clotting (Table II, Experiment 14). This does not seem to be in agreement with the views of

Waldschmidt-Leitz and collaborators (20), who regard the coagulation of blood as a proteolytic process which according to them is accelerated by trypsin kinase and inhibited by a number of proteolytic substrates, *e.g.* clupein, thymus histone, etc.

Both cellulose sulfuric acid and polyvinyl sulfuric acid (Table I, Experiments 1 to 3) showed the same, very high activity, whereas polyvinyl alcohol proved entirely inactive (Table II, Experiment 4). It was interesting to find that an inhibitor substance, a galactan sulfuric acid ester, occurs in the alga, *Iridex laminarioides* (12) (Table I, Experiment 4).

The data on the activity of heparin preparations given in Table I (Experiments 5 to 7) show that, while commercial heparin samples are less active than the synthetic anticoagulants, it is not difficult to obtain heparin preparations from lungs or liver, which are considerably more active than any polysaccharide sulfuric acid examined up to now. This preparation (Table I, Experiment 7) is by no means a homogeneous substance, and can be further purified with accompanying increase in activity. The question arises as to whether this enormous difference in activity between the synthetic and natural anticoagulants is in harmony with the view that the latter are sulfuric acid derivatives of chondroitin-sulfuric acid.

As a result of the experiments here described, which in general are in good agreement with the findings of Jorpes and Bergström, one can conclude that, as the matter now stands, the following properties seem to be necessary for a substance which is to act as inhibitor of blood clotting. It must be water-soluble, of high molecular weight, and it must contain combined sulfuric acid or possibly other acid groups of similar strength.

EXPERIMENTAL

Preparation of Material

Cellulose Sulfuric Acid—This acid was prepared according to Traube and collaborators (10) by treating dry, defatted cotton suspended in pyridine with chlorosulfonic acid. By this method somewhat better results were obtained than by that of Gebauer-Fülneegg and collaborators (11) who carried out the reaction at a higher temperature. The pyridine salt obtained was converted into the sodium salt, a white amorphous powder giving highly

viscous solutions with water. This preparation has a slightly higher inhibitor activity than commercial heparin (Hynson, Westcott and Dunning, Lot 130) (Table I, Experiment 1) and seems to correspond to the substance tested by Bergström (9).

This product could be further purified. 1 gm. of the sodium salt was dissolved in 20 cc. of water; the solution was made slightly alkaline to phenolphthalein with sodium hydroxide and centrifuged in the angle centrifuge at 3000 R.P.M. for 15 minutes. The precipitate was washed with slightly alkaline water and with alcohol. This substance after drying weighed 110 mg. and formed a white horn-like material. On treatment with water it formed what was apparently a clear aqueous solution. That the substance was not really dissolved is shown by the fact that it precipitated on being centrifuged. This product, which is entirely inactive in the inhibition of blood clotting (Table II, Experiment 5), gave on analysis values that were quite near those required by the *sodium salt of a cellulose monosulfuric acid*.

$C_6H_7O_5SNa$ (264.1).	Calculated.	S 12.2, Na 8.7
	Found.	" 12.9, " 9.4
	"	S:Na, 1:1.02

To the solution from which the insoluble material had been removed 3 volumes of alcohol were added. The precipitated material was centrifuged and washed with alcohol. After two more reprecipitations a white water-soluble powder was obtained which weighed 450 mg. This substance is extremely active as inhibitor of blood clotting. In our method it was found to have 250 inhibitor units per mg., and it was about 4 times as active as a commercial heparin preparation of 5 Howell units (see Table I, Experiment 2). Its sulfur content points to a *sodium salt of a cellulose disulfuric acid*.

$C_6H_5O_{11}S_2Na_2$ (366.2).	Calculated.	S 17.5, Na 12.6
$C_6H_7O_{14}S_2Na_2$ (468.3).	"	" 20.5, " 14.7
	Found.	" 17.0, " 14.4
	"	S:Na, 1:1.2

Polyvinyl Sulfuric Acid—Polyvinyl alcohol is soluble in water. It showed no activity whatsoever in the inhibition of blood clotting when examined in a 1.03 per cent solution (Table II, Experiment 4). 1.04 gm. of the dried material were suspended in 24 gm. of dry

pyridine, and 6 gm. of chlorosulfonic acid were slowly added to the chilled mixture. After the reaction had slowed down, the mixture was kept at 70° for 1½ hours. The mother liquor was decanted from the reaction product which formed a rubber-like, very elastic material. It was taken up in 20 cc. of water, made alkaline with 2 N KOH, and 80 cc. of alcohol were added to the solution. After cooling, the precipitate was removed by centrifugation and dissolved in 75 cc. of warm water. The solution was centrifuged, the supernatant liquid filtered through cotton, and the product precipitated by the addition of 150 cc. of alcohol. After two more precipitations 1.85 gm. of the *potassium salt of polyvinyl sulfuric acid* were obtained as a white water-soluble powder which was highly active as inhibitor of blood clotting (250 inhibitor units per mg.; see Table I, Experiment 3).

(C ₂ H ₃ O ₄ SK) _x (162.2).	Calculated.	S 19.7, K 24.1
	Found.	" 18.1, " 25.9
	"	S:K, 1:1.1

Measurement of Inhibition Activity

In all experiments the measurement of inhibitor activity was carried out according to the method described in the preceding paper (21). The substances were dissolved in physiological saline, the maximum concentration in which the compounds classified as inactive were tested being between 1.2 and 10 mg. per cc. of plasma. In the case of active substances much lower concentrations could be used.

The results obtained with the various substances tested are summarized in Tables I and II, the number of inhibitor units per mg. being given in each case.

In conclusion we should like to express our indebtedness to our colleagues who have kindly given us samples of some of the compounds tested in the course of this work, *viz.* Dr. W. Z. Hassid of the University of California, Berkeley, and Doctors H. T. Clarke, M. Heidelberger, K. Meyer, F. Cortese, and G. C. H. Stone of this University. A sample of highly purified β -amylose was given us by the late Dr. T. C. Taylor. We are also indebted to Hoffmann-La Roche, Inc., and to Hynson, Westcott and Dunning, Inc., for samples of heparin.

SUMMARY

1. A number of substances of high molecular weight, both of neutral and acidic properties, have been examined as to their activity as inhibitors of blood clotting.

2. The preparation of cellulose mono- and disulfuric acids, and of polyvinyl sulfuric acid is described; the inhibitor activity of these compounds is discussed.

3. A naturally occurring galactan sulfuric acid ester has been found active in the inhibition of blood clotting.

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BLOOD LACTIC ACID FOLLOWING THE ADMINISTRATION OF INSULIN IN CATS WITHOUT THE ADRENAL MEDULLA

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The following study is concerned with the behavior of lactic acid in the blood after the administration of insulin. In view of the sensitivity of the lactic acid mechanism to epinephrine injections, animals were desired that would give no excitement reactions to the necessary drawing of blood samples without an anesthetic. This condition may be achieved to some extent in normal animals by training, but to eliminate a possible epinephrine interference, we decided to work upon animals from which the adrenal medulla in both glands had been removed by operation.

We had available for study three cats from which one adrenal medulla in each had been removed by dissection, leaving the cortex present. In a subsequent operation the other gland in each cat was entirely removed.

At the conclusion of the experiment histological examination of serial sections of the glands showed the following: in one animal (Cat 107) no medullary tissue was found; in the second (Cat 105) an area of medullary tissue measuring 0.3 mm. \times 0.5 mm. was found; in the third (Cat 101) a remnant measuring 1 mm. \times 0.4 mm. was present.

These animals had been in use in the laboratory for a period of 4 months. They were in excellent general health. The average weight was about 4 kilos. Previous to use the animals were fasted for from 18 to 20 hours. They were thoroughly accustomed to the handling attendant upon the drawing of blood samples at short intervals and showed no resentment to the treatment. Blood was drawn from the femoral or saphenous vein without the use of an anesthetic. Insulin (Eli Lilly) was given by intravenous injection,

following the taking of the initial sample. After the injection of insulin the animals were very quiet and showed practically no muscular activity until the period of onset of convulsions, if they occurred. Blood samples were taken at intervals of 15, 30, 60, 90, 120, 180, 240, and occasionally of 300 minutes after the injection of insulin. The blood was collected in dry potassium oxalate containing 50 per cent of sodium fluoride.

The method for the determination of blood sugar was that of Shaffer and Hartmann for 1 cc. of blood. The method for the determination of lactic acid was a modification of that of Friedemann, Cotonio, and Shaffer (Scott, 1936).

TABLE I
Lactic Acid per 100 Cc. of Blood

	Copper-lime treatment	Without copper-lime
	<i>mg.</i>	<i>mg.</i>
Venous	34.9	34.0
	27.5	28.9
	24.5	23.6
	14.9	16.4
Arterial, under ether	27.7	25.4
	29.3	32.0
	34.2	33.5
Average.....	27.4	27.7

Following the precipitation of the proteins by sulfuric acid and sodium tungstate, the filtrate was cleared of glucose by the use of copper sulfate and lime (Van Slyke, 1917).

Boyland (1928) suggested that glucose does not interfere in the determination of lactic acid even when permanganate is used as the oxidizing agent. We compared the values for lactic acid with and without the use of copper sulfate and lime in the blood of a normal cat, drawn without anesthesia from a leg vein, and also in arterial blood from a cat under ether anesthesia (Table I).

Similar results have been reported by Cook and Hurst (1933) with the Clausen method with dilute permanganate and sulfuric acid (1922).

Notwithstanding these observations that would lead to the conclusion that sugar does not interfere in the determination of

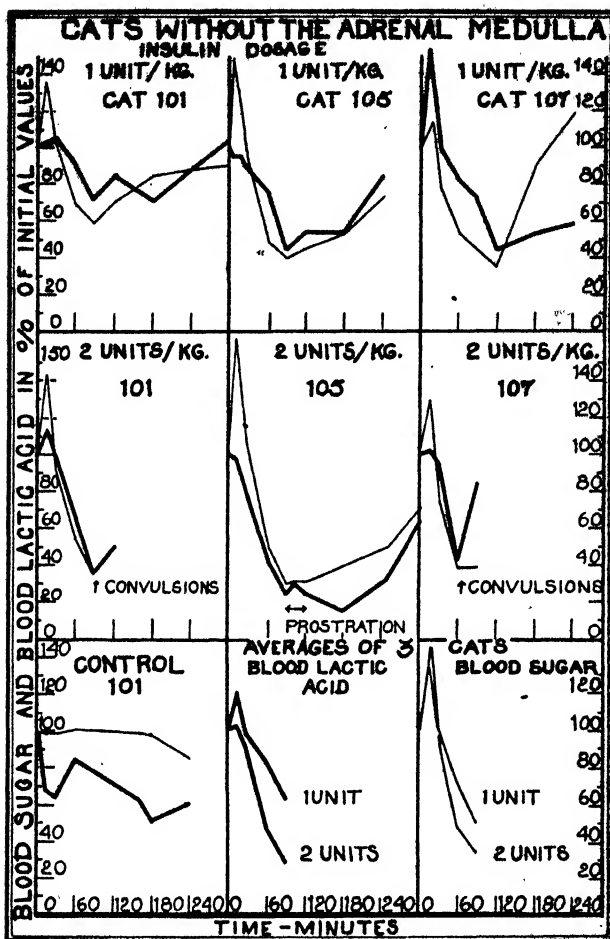


FIG. 1. Blood sugar and blood lactic acid expressed as percentages of the initial values. The heavy line represents lactic acid; the light line, blood sugar. The curves represent changes in the percentage values of the blood sugar and the blood lactic acid in cats without the adrenal medulla, following the injection of 1 and of 2 units of insulin per kilo of body weight. One control is given. The average values are also shown. The 90 minute point in the blood lactic acid curve, following the injection of 2 units of insulin, is the average for two cats (Nos. 101 and 105); the third cat was in convulsions.

lactic acid even when permanganate is used as the oxidizing agent, all of the work was done with filtrates from which presumably the glucose had been removed by the standard method.

The precision of the method for lactic acid determination as carried out here was satisfactory, since the average difference between the duplicates was 1.5 mg. per 100 cc. of blood.

TABLE II

Concentration of Lactic Acid in Mg. per 100 Cc. of Blood Following Administration of Insulin in Cats without the Adrenal Medulla

The figures in this table represent averages of duplicate analyses of 1 cc. blood samples except where noted.

Time	Cat 101		Cat 105		Cat 107		
	Dosage of insulin in units per kilo						
	1 unit	2 units	None	1 unit	2 units	1 unit	2 units
<i>min.</i>							
0	13.9	15.3	20.9	14.4	25.3*	18.2	21.4
10				13.7			
15	14.1	17.4	13.9		24.7*	28.0	21.9
20				13.5			
30	14.7	15.0	13.2	12.9	20.2	18.0	20.2
60	12.6	9.9	17.5	11.0	10.5	15.1	8.7†
90	9.9	5.3†		6.3	6.4	13.0	17.8
120	11.7	7.7		7.8	6.5	8.1*	
162			13.4				
180	9.9		10.9	7.7	3.8‡	9.6	
240	12.2		13.0	11.9	8.0*	10.7*	
300	14.5				15.9		

* One determination only.

† Convulsions occurred shortly after taking this sample.

‡ The animal was prostrated at this time.

The blanks were 0.8 ± 0.06 cc. of 0.001 N iodine in 5 per cent potassium iodide on ten determinations.¹ This is the iodine equivalent given by Friedemann and Graesser (1933). Using an all-glass apparatus, we obtained the same blanks. All of the chemicals that were used were Merck's reagent chemicals. The small deviation of the blanks is of greater importance than their absolute values.

¹ Deviations are expressed as mean deviations (Scott, 1927).

The average initial blood sugar was 86 ± 8.9 mg. per 100 cc. of blood. The average initial blood lactic acid was 18.0 ± 2.2 mg. per 100 cc. of blood.

Since this type of animal material is limited in quantity, all of the data on lactic acid, which consist of six experiments upon three animals, three experiments with 1 unit of insulin and the same number with 2 units per kilo of body weight, are presented here. The absolute values are given in Table II. The percentage values in terms of the initial values are given in Fig. 1. One control in which no insulin was injected is included. The blood sugar values appear in Fig. 1 only.

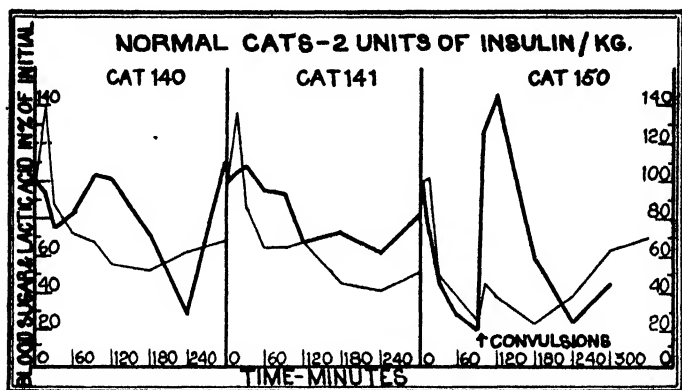


FIG. 2. Percentage changes in blood lactic acid and in blood sugar in three normal cats. The heavy line represents lactic acid; the light line, blood sugar.

Study of Table II and of Fig. 1 shows that there is a marked similarity between the effect of insulin upon the blood sugar and upon the lactic acid. Expressing the values as percentages of the initial values makes the similarity very striking (Scott and Dotti, 1932). An initial rise is characteristic of the blood sugar curve in the cat. A similar initial rise in blood lactic acid sometimes occurs.

The injection of 2 units of insulin causes a more rapid and a more pronounced fall in the blood lactic acid in all three of the cats than that produced by 1 unit. This is shown in Fig. 1 by the averages for the first 60 minutes. There is a drop of 17 per cent with 1 unit, and with 2 units the drop is 53 per cent.

The accepted opinion at the present time is that insulin has no direct effect upon lactic acid in normal animals (Himwich, 1932; Cori, Cori, and Buchwald, 1930). The opinion was advanced somewhat earlier, however, that a regular and inverse relationship existed between sugar and lactic acid in the blood under the action of insulin (Briggs *et al.*, 1924).

For comparison with the work offered here on the cats without the adrenal medulla we present also three curves showing percentage changes in blood sugar and in blood lactic acid in normal cats following the injection of insulin. The experimental conditions were the same. The blood was drawn at short intervals following the injection of 2 units of insulin per kilo of body weight from the leg vein without anesthetic. The preliminary period of fasting was from 18 to 20 hours, except in Cat 150 in which the period without food was more than 24 hours. The curves are shown in percentage changes of the initial values (Fig. 2). The absolute values of the initial samples are given below.

Cat No.	Lactic acid per 100 cc. blood	Sugar per 100 cc. blood
	<i>mg.</i>	<i>mg.</i>
140	28.0	81.6
141	18.4	89.6
150	39.2	94.4

From these curves it is indeed very difficult to interpret the changes that occur in blood lactic acid following the injection of insulin in normal animals. The observations on Cat 140 might support the opinion of an inverse relationship (Briggs *et al.*, 1924), if the samples happened to be taken at the proper moments. An indefinite reaction is obtained in Cat 141. In Cat 150, however, there appears to be a typical insulin effect. During the initial 90 minute period both sugar and lactic acid fell proportionately. Following the incidence of the convulsions, which occurred at 107 minutes after the injection, there was a marked increase in lactic acid in the blood and a smaller increase in blood sugar. The rise in blood lactic acid reached its peak within 13 minutes after the convulsions. The insulin effect then reappeared and lasted for 120 minutes. Thereafter, there was a recovery in both sugar and lactic acid.

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In animals without the adrenal medulla there seems to be a demonstrable effect of insulin upon blood lactic acid and this is more pronounced with increased dosage. In these animals the lactic acid and the sugar metabolism seem to be intimately related and similarly affected by insulin.

If further work substantiates the implications of these observations, it will, apparently, be necessary to make some fundamental modifications in the current conception of the rôle of lactic acid in carbohydrate metabolism.

SUMMARY

In three cats without the adrenal medulla the blood lactic acid and the blood sugar curves following the injection of insulin are very similar.

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THE REACTION OF CYANIDE WITH THE HEMOCYANIN OF LIMULUS POLYPHEMUS

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Kobert (1) observed that cyanide reacts with oxyhemocyanin so as to remove oxygen, forming a colorless complex. The stoichiometric aspects of this reaction have been studied on hemocyanin prepared from the blood of the horseshoe crab, *Limulus polyphemus*. It has been found that cyanide reacts with oxyhemocyanin to form a cyanhemocyanin, which is a fairly stable compound, but that under certain conditions the reaction may be qualitatively reversed. Since cyanide removes oxygen from oxyhemocyanin (1), it is assumed that cyanide reacts with the prosthetic group so that it can no longer hold oxygen. Evidence will be presented to show that the combining ratio of cyanide to copper, in the case of *Limulus* hemocyanin, is four cyanide groups to 2 atoms of copper.

Methods

In studying the reaction of hemocyanin with cyanide, it is desirable to know accurately the amount of copper present, as a measure of the number of prosthetic groups. The precise determination of copper was made by the micromethod of Schoorl and Begemann (2), simplified by Redfield, Coolidge, and Shotts (3). Estimation of copper was also made by determining the refractive index of purified hemocyanin solutions (4).

Hemocyanin was purified by dialysis of serum and precipitation of the salt-free solution at the isoelectric point (3). The hemocyanin was washed by repeated precipitations at the isoelectric point, and the final precipitate of hemocyanin was dissolved in a minimum of 0.01 N NaOH, which gave approximately a 3 per cent solution.

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Oxyhemocyanin has an intense blue color in solution. When treated with KCN, it forms a cyanhemocyanin which is colorless. The color of hemocyanin solutions has been taken as an indication of the degree of oxygenation of the protein and was used by Pantin and Hogben (5), to construct oxygen dissociation curves. Redfield (6) has studied the absorption of light by hemocyanin solutions and has developed a spectrophotometric method for measuring the degree of oxygenation of such solutions. Since cyanide is assumed to replace oxygen in oxyhemocyanin, a measure of the degree of oxygenation gives by difference a measure of the progress of the cyanide reaction. By this means it was possible to follow the combination of cyanide with oxyhemocyanin. All measurements on the spectrophotometer were made with light of a wave-length of $590\text{ m}\mu$, where *Limulus* hemocyanin has maximum absorption. The equilibration of hemocyanin solutions with KCN was carried out in tonometers of the type described by Redfield (6), so that measurements of the color could be readily made with the spectrophotometer.

EXPERIMENTAL

2 ml. of the purified solution were introduced into a tonometer and the volume increased to 6 ml. with distilled water. When equilibrium was reached, the extinction coefficient was measured with the spectrophotometer, which gave a value for the oxygenated hemocyanin at this particular concentration. Additional portions of 2 ml. of the hemocyanin solution were treated with successively increasing concentrations of KCN solution and were allowed to stand for 48 hours at 20° until equilibrium had been reached. The solutions were diluted in each case to 6 ml. with distilled water and the tonometers stoppered. At the end of the 48 hour period the extinction coefficients of the various samples were measured. The results of this experiment are summarized in Table I.

The uncombined KCN is obtained by correcting for the amount of cyanide used up in the formation of cyanhemocyanin, assuming that 1 atom of copper is equivalent to 2 cyanide ions. The justification of this assumption is discussed below. When the uncombined KCN is plotted against the percentage of cyanhemocyanin, a sigmoid curve, characteristic of oxygen dissociation

curves for respiratory proteins, is obtained. The reaction of oxyhemocyanin with cyanide may be represented as follows:



where RCu_2O_2 represents the quantity of oxyhemocyanin reacting with n moles of cyanide to form the cyanhemocyanin, $\text{RCu}_2(\text{CN})_n$. When the mass law is applied to this equilibrium, the following linear equation is obtained, "when the oxygen tension is constant.

$$\log \frac{\text{RCu}_2(\text{CN})_n}{\text{RCu}_2\text{O}_2} = \log k + n \log \text{KCN} \quad (2)$$

where k is the equilibrium constant.

TABLE I
Formation of Cyanhemocyanin in Presence of Various Amounts of Potassium Cyanide

KCN (total added)	KCN (uncombined)	Cyanhemocyanin
<i>mM</i>	<i>mM</i>	<i>per cent</i>
0.00	0.00	0.0
0.51	0.49	4.5
0.70	0.59	15.3
0.84	0.73	21.8
1.20	0.94	44.0
1.36	1.09	49.2
1.70	1.29	73.2
2.05	1.57	83.6
2.56	2.03	92.9
3.41	2.84	100.0

Cu = 0.283 mM in each case.

Equation 2 may be plotted as a straight line with $\log \text{RCu}_2(\text{CN})_n / \text{RCu}_2\text{O}_2$ as ordinate and $\log \text{KCN}$ as abscissa, in which case k will be given by the intersection with the abscissa, and n will be the slope of the line. The concentration of KCN must be corrected for the amount used in the formation of cyanhemocyanin, according to the value of n . Upon experimenting with different values of n , it was found that the above conditions are satisfied when n is equal to 4. The line corresponding to Equation 2 and which fulfils these conditions is plotted in Fig. 1.

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This information indicates that the value of n in Equation 1 is equal to 4.

Three similar experiments were carried out with *Limulus* serum instead of the salt-free hemocyanin. The molecular concentration of hemocyanin was varied in each experiment. Sigmoid curves were obtained as before. When the mass law is applied, as above, straight lines resulted in each case, having a slope of 4 ($n = 4$);

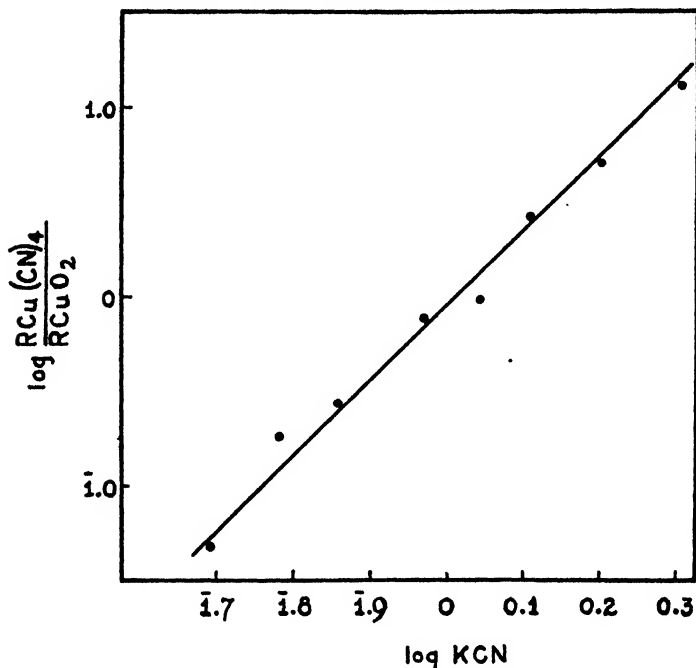


FIG. 1. Graphical representation of Equation 2 with data from Table I; Cu should be divalent.

the equilibrium constants varied with the dilution of the serum. This information serves to verify the relationship of two cyanide groups to 1 copper atom.

Craifaleanu (7) found that solutions of *Octopus vulgaris* cyanhemocyanin, which had not been treated with an excess of cyanide, recovered the blue color in the presence of oxygen. Thus he concluded that cyanhemocyanin was an unstable compound, and that oxygen gradually replaced the cyanide. This phenomenon

could not be demonstrated with *Limulus* hemocyanin. Both solutions of cyanhemocyanin which did not contain an excess of KCN, and also some in which the reaction had only gone part way to completion, were allowed to stand exposed to the air for a period of weeks without a measurable change in their color. Similar solutions in which the pressure of oxygen was increased showed no corresponding increase in the color. The hydrogen ion concentration of these solutions was estimated to be about pH 9. At this alkalinity HCN is only slightly dissociated. It seemed possible that on lowering the acidity to pH 7, where the HCN would be more dissociated, evacuation of the tonometers would draw off some of the HCN gas. Then, increasing the pressure of oxygen might bring about a reversal of the reaction. Such experiments were set up. The pH was lowered by inhaling CO₂ into evacuated

TABLE II
Dialysis of KCN-Hemocyanin Solution

Condition of solution	Log tangent of angle	Oxyhemocyanin
		<i>per cent</i>
Before treatment with KCN.....	1.632	100
2 days after treatment with KCN.....	1.119	0
3 wks. " dialysis.....	1.428 -	60

tonometers. The pressure of oxygen was increased to 5 atmospheres. Upon equilibration there was no measurable change in color.

A qualitative demonstration of a reversal of the reaction, however, was given by dialysis of a cyanhemocyanin solution containing a slight excess of cyanide. 15 ml. of purified oxyhemocyanin solution containing 0.81 mg. of copper were diluted with 20 ml. of distilled water and treated with 13.3 mg. of KCN, which was slightly more cyanide than was necessary to make the reaction go to completion. The mixture was allowed to equilibrate for 48 hours. It was then transferred to a collodion membrane and dialyzed under reduced pressure for 3 weeks. Dialysis was carried out with distilled water at first and then with 0.001 N NaOH. Table II indicates the progress of the reaction at various intervals.

In both serum and salt-free solutions an amount of cyanide

in excess of 4 equivalents was necessary to replace all of the oxygen from the oxyhemocyanin. It was of interest to know if all of the cyanide is combined with the hemocyanin, or if some is free in solution. Analyses for free cyanide in the equilibrium mixture were made by titration with silver nitrate (8). The titrations were carried out in a dark room. Light was projected vertically through the solutions which were then viewed horizontally against a dark background. Owing to the scattering of light by protein, the solutions had a slight cloudy appearance before any silver nitrate was added. This made it difficult to observe the end-point of the KCN-AgNO₃ reaction. The analyses

TABLE III
Analyses for Cyanide

KCN solutions	0.005 N AgNO ₃ (slightly alkaline)
	ml.
1.33 mg. KCN-aqueous solution	1.92
	1.91
1.33 " KCN + hemocyanin solution containing 0.108 mg. Cu; titrate immediately	2.10
	2.18
	2.10
	2.15
Same; equilibrate 1 day, then titrate	0.82
	0.50
" " 2 days, " "	0.65
	0.70
	0.52

were, therefore, not very accurate, but there is sufficient accuracy to demonstrate that some cyanide is free in the equilibrium mixture.

2 ml. portions of salt-free hemocyanin solution containing 0.108 mg. of copper were treated with KCN, but in no case was the KCN in excess. Titrations with AgNO₃ were made immediately after the addition of cyanide, and other titrations were made when equilibrium was reached. The results are expressed in Table III. They indicate that approximately one-fourth of the cyanide introduced is free in the equilibrium mixture.

A knowledge of the relative concentrations of oxygen and

cyanide present at a point in the equilibrium of oxyhemocyanin with cyanide, where the concentration of oxyhemocyanin equals the concentration of cyanhemocyanin, should give a value of the relative affinity of hemocyanin for these two substances. This method of determining relative affinity has been used by Henderson and Haggard (9). By use of the data from the experiment described above and the solubility of oxygen in distilled water, it is found that the affinity of salt-free hemocyanin for oxygen is approximately 5 times that for cyanide.

SUMMARY

The stoichiometric aspects of the reaction of KCN with *Limulus* hemocyanin have been studied. It is found that cyanide reacts with oxyhemocyanin so as to remove the oxygen, forming a rather stable cyanhemocyanin. By dialysis, the reaction may be reversed. The reaction expressed graphically gives a sigmoid curve. Application of the mass law indicates that the reaction takes place as follows:



The relative affinity of salt-free hemocyanin for oxygen and cyanide is approximately five to one respectively.

I wish to thank Professor Alfred C. Redfield for the suggestion of this problem and for the use of his laboratory and equipment.

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EFFECT OF DIET ON PHOSPHATE COMPOUNDS IN THE LIVER OF THE DOG*

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The liver, because of its capacity for storage of foodstuffs, may undergo great changes in chemical composition. Glycogen, for example, may vary from less than 1 to more than 20 per cent (1), and fat may vary from less than 3 to more than 40 per cent (2). The liver is actively involved in the intermediate metabolism of foodstuffs; it maintains a normal level of blood sugar in the animal by alternate release and storage of carbohydrate (3); it deaminizes amino acids and produces urea, the chief waste product of nitrogen metabolism in the mammal (4); and it plays a definite part in the metabolism of fat, although the nature of this participation is controversial.

Because phosphorus occurs in the organism not only as inorganic phosphate but also in many organic compounds, including carbohydrates, proteins, and lipids, the changes of phosphates which occur in an organ so involved in the metabolism of all the major foodstuffs as is the liver should be of particular interest. Some studies of these phosphate compounds in the hepatic tissue of dogs maintained on a variety of different diets have now been carried out. Of primary interest to us were the acid-soluble phosphates because such compounds in muscle tissue have been shown by many workers to be of importance in the metabolism of carbohydrates. As these acid-soluble phosphate compounds constitute only a third of the total quantity of phosphates in the liver, direct determinations of phospholipid phosphorus and

* Taken from a thesis submitted by Eunice Flock to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

indirect determinations of residual phosphorus were also made. Analyses were carried out for glycogen, fat, and water with the idea of correlating possible changes in the phosphates with the very large changes that can be produced in these constituents of the liver.

The chief variants in these studies were in the dietary conditions, which included a normal kennel diet, a standard balanced diet, and high protein, high carbohydrate, and high fat diets as well as periods of fasting. In a few cases the effect of injecting glucose was studied. All animals were fasted for 18 hours prior to removal of the liver, unless otherwise stated in the tables.

Methods

Dogs were anesthetized with amytal or ether. One or more lobes of the liver were quickly removed and dropped into a freezing mixture of carbon dioxide ice and alcohol. The time interval between induction of anesthesia and removal of the tissue was short, no differences in the phosphate compounds as a result of differences in anesthesia being noted.

Preparation of Acid-Soluble Extract—This was carried out in the cold room, as described by Pollack, Flock, and Bollman in a study of phosphate in muscle (5). The frozen tissue was ground in a fine meat grinder. Duplicate samples of approximately 10 gm. each were weighed and transferred to 250 cc. centrifuge bottles which contained a few ounces of solid glass beads. Enough 5 per cent trichloroacetic acid was added to make a final volume of 100 cc., after allowing for a 75 per cent water content in the tissue. The bottles were then put in the shaking machine and the tissue was extracted for 15 minutes. The tissue becomes so finely pulverized that a short centrifugation is desirable prior to filtration of the extracts by suction. Aliquots were ashed with sulfuric acid and superoxol for the determination of the total acid-soluble phosphorus. Large aliquots were used for subsequent fractionation.

Preparation of Alcohol-Ether Extract—The residues from the acid extraction were washed on suction filters with several portions of trichloroacetic acid and were kept there until quite dry. They were then returned to the centrifuge bottles. To each were added 100 cc. of a 3:1 alcohol-ether mixture. 2 hours at room temperature were allowed for complete extraction, and during 1 hour of

this time the bottles were shaken on the shaking machine. The bottles were then centrifuged for a few minutes and the extract was decanted. Aliquots were ashed for total phospholipid phosphorus. Lecithin and cephalin are readily soluble in cold mixtures of alcohol and ether but sphingomyelin is claimed to be insoluble; Fränkel and Löhr (6), however, have shown that this occurs only to the extent of 0.03 per cent in hepatic tissue, which in terms of phosphorus would be only 1.2 mg. per 100 gm. and would thus be a negligible error in our determinations.

Cold extraction is very easy and convenient to carry out as compared with a prolonged hot extraction, and this was a matter of first importance as so many analytical procedures were involved in these experiments. The advantage of carrying out alcohol-ether extraction subsequent to acid extraction lies in the preliminary removal of certain acid-soluble phosphates which are also alcohol-soluble. Teorell and Norberg (7), since the beginning of this work, have reported using preliminary trichloroacetic acid extraction prior to hot extraction in the Soxhlet apparatus for phospholipid, which they wished to determine by phosphorus content.

Total Phosphorus—Samples of liver, 1 or 2 gm., were weighed, dried to constant weight at 80° for water content, and ashed with fuming nitric acid for total phosphorus content (8).

Residual Phosphorus—This was obtained by difference from the sum of the acid-soluble and alcohol-ether-soluble phosphorus and the total determined on the fuming nitric acid ash. Thus the actual values are affected by experimental errors in three determinations and must be considered of only relative significance. Jorpes (9) has shown that the residual phosphorus is practically equivalent to nucleic acid phosphorus. Residual phosphorus would, of course, also include the phosphoproteins.

Fractionation of Acid Extract—The method of fractioning the acid-soluble phosphates was essentially the one of barium separation described by Eggleton and Eggleton (10) for muscle tissue and used in modified form by Pollack, Flock, and Bollman. Eggleton and Eggleton stated that they were unable to use it on hepatic tissue owing to interference from yellow pigment. In using dog livers and taking precautions to freeze the tissue and extract it at 0° we have encountered no difficulty from this source.

There is a yellow tinge to the extract, but at the dilution used no more so than in a similar extract of cardiac tissue. The real difficulty associated with this fractionation appears whenever the glycogen content of the liver is 2.5 per cent or higher; then the acid extract is opalescent with glycogen and the usual precipitation procedures are ineffective.

Precautions were taken to keep the extracts cold during fractionation. This was done by carrying out the process in the cold room or by keeping the tubes immersed in ice water. Aliquots of 40 cc. of the acid extract were partially neutralized with 0.5 cc. of saturated sodium hydroxide and were then treated with powdered barium oxide until alkaline to phenolphthalein. 10 minutes were allowed for settling of the precipitates before centrifugation and decanting of the clear supernatant liquid into a 50 cc. volumetric flask. Each precipitate was dissolved with 2 cc. of 5 per cent trichloroacetic acid and 3 cc. of water and then reprecipitated by neutralization with barium oxide. After centrifugation the supernatant liquid was added to that previously obtained. Distilled water was added to make a total volume of 50 cc. This fraction will be subsequently referred to as Fraction A or the barium-soluble phosphates.

The phosphates precipitated by the barium treatment were dissolved with a few drops of 8 N hydrochloric acid and water, transferred to a 50 cc. volumetric flask, and made up to volume with water. This fraction will be referred to as Fraction B or the barium-insoluble phosphates.

When the trichloroacetic acid extract was opalescent with glycogen, a modification of this fractionation had to be used. Glycogen holds more or less of the barium-insoluble phosphates in colloidal suspension, and thus must be removed before any fractionation is possible. This may be done by adsorption on mercuric sulfide, as described by Kerly and Ronzoni (11) for muscle extracts. More recently, Bott and Wilson (12) have used a similar procedure for removal of glycogen from liver extracts used for lactic acid analyses. To insure flocculation of the mercuric sulfide they heated their extracts. Because of the lability of at least one of the phosphate compounds, heating could not be used in our experiments. In our early experiments a solution of 15 per cent mercuric chloride in 0.5 N hydrochloric acid was added in small portions to the extract, which was then subjected to a

continuous stream of hydrogen sulfide until mercuric sulfide was coagulated. In later experiments finely powdered mercuric chloride was added directly to the extract, which was thoroughly shaken during treatment with hydrogen sulfide. The salt was added in small quantities until adsorption of the glycogen was completed. If during filtration the apparently well flocculated mercuric sulfide suddenly changed over to the colloidal state, it was necessary to add mercuric chloride until complete precipitation again occurred, when the sulfide could be readily removed by centrifugation and filtration. The excess hydrogen sulfide was removed by aeration and the extract was fractionated with barium oxide as just described. The extracts were kept at 0° throughout this process for the removal of glycogen. When this procedure is used with care, no loss in phosphate compounds is encountered.

The final analyses of the various phosphate compounds were carried out by the method of Fiske and Subbarow (13). This method could be used directly for the inorganic phosphate; for the organic compounds a preliminary conversion to the inorganic form was made either by acid hydrolysis or ashing. As mentioned before, for ashing the whole tissue fuming nitric acid was used; for ashing the various extracts or fractions thereof sulfuric acid and superoxol were used. When the final reagents were added for the phosphate determinations, allowance was made for the sulfuric acid used in hydrolysis or ashing so that the final concentration of reagents was that described by Fiske and Subbarow.

Fraction B was analyzed for inorganic phosphate, phosphate hydrolyzable in 15 minutes, and total barium-insoluble phosphate. In addition, in some of the experiments aliquots were hydrolyzed for periods of 1, 2, and 4 hours. In all hydrolyses 3 cc. of the solution and 3 cc. of 2 N sulfuric acid were combined and heated at 100° for the specified time and then cooled in ice water. The amount of inorganic phosphate thus hydrolyzed was measured. Precautions were taken to keep the fraction cold prior to hydrolysis. The phosphate hydrolyzable in 15 minutes found in this fraction in muscle is adenosine triphosphate. That the liver nucleotide must be similar to that from muscle was suggested by our preliminary studies, in which we obtained a positive Bial's test for pentose in this fraction and isolated adenine as the picrate following acid hydrolysis.

Fraction A was analyzed for labile, alcohol-insoluble, and total

barium-soluble phosphates. In muscle tissue the very labile phosphocreatine occurs in this fraction. The test for phosphocreatine, the production of inorganic phosphate on standing with the molybdate reagent for 30 minutes at room temperature, was always negative in the liver. Occasionally false positives were obtained when the glycogen content was around 2 or 2.5 per cent. In such cases the extract may not appear quite opalescent enough to require mercuric sulfide treatment and yet there may be sufficient to hold up a small amount of the inorganic phosphate, which might be mistaken for a labile phosphate compound were it not for the fact that the sum of the inorganic phosphates obtained in Fractions A and B always equalled the total inorganic phosphate of the original extract. Whenever the effect of glycogen was eliminated, a clean separation was obtained and the test for labile phosphates in Fraction A was completely negative. The occurrence of phosphocreatine in rabbit liver reported by Takahisa (14), may possibly be explained on the basis of non-removal of glycogen.

To obtain the alcohol-insoluble phosphate an aliquot of 24 cc. of Fraction A was precipitated with 120 cc. of ammoniacal alcohol, was allowed to stand overnight in the ice box, and was then centrifuged. The precipitate was dissolved with a few drops of 8 N hydrochloric acid and water, the barium was removed with sulfuric acid, and the volume was made up to 15 cc. 3 cc. were then ashed for phosphate content. Cori and Cori (15) used such a procedure for the determination of hexosemonophosphate in muscle following a preliminary breakdown of phosphocreatine. Whether or not hexosemonophosphate occurs in liver is not known. That the substance precipitated in this manner in liver is largely α -glycerophosphoric acid is very probable. Fiske and Subbarow have isolated this as the calcium salt by precipitation of liver extracts with calcium chloride and calcium hydroxide and alcohol added to make a final concentration of 80 per cent. They found that glycerophosphoric acid constituted about a third of the acid-soluble phosphates. Our procedure for its quantitative estimation was very similar to their method of isolation, and in our normal animals it occurred in a similar proportion of the total. The qualitative tests which they used for its detection, such as the production of acrolein upon heating with potassium acid sulfate and the positive Denigès reaction with phenols (16), were positive

with our precipitate. The barium-alcohol-soluble phosphate was obtained by difference from the value for the alcohol-insoluble and total phosphate of Fraction A. This compound has not been identified. Booth (17) has recently reported that there is definite evidence for a very soluble choline ester in liver like the one which he has isolated from the kidney; it contains phosphoric acid and what appears to be sphingosine. How it compares with our compound remains to be shown. In some experiments aliquots of Fraction A were hydrolyzed for 15 minutes, and for 1, 2, and 4 hours, as described for Fraction B.

Similar hydrolyses have also been carried out on the original acid extract. All the values given in Tables I to VI for the phosphate analyses of the extracts are averages of the determinations made on aliquots from the two original samples of tissue used for extraction. All values are expressed in terms of mg. of phosphorus per 100 gm. of moist tissue. Analyses for glycogen, water, and total phosphorus were made on duplicate samples of tissue and the averages are used in Tables I to VI, glycogen being expressed in terms of glucose.

Fatty Acid Determination—5 gm. of normal hepatic tissue or 2 to 3 gm. of grossly fatty hepatic tissue were weighed and analyzed according to a modification of Kumagawa and Suto's method (18). The tissue was digested for 2 hours on the steam bath with 40 cc. of 25 per cent potassium hydroxide and 10 cc. of ethyl alcohol. After cooling and strong acidification with hydrochloric acid, the fatty acids were extracted with several portions of ethyl ether. This was then evaporated, and the residue was allowed to stand for several hours in contact with petroleum ether for complete extraction of the fatty acids. The extract was filtered, the petroleum ether was evaporated, and the fatty acids were then weighed.

Results

Values for total phosphates in the livers of normal dogs maintained on the regular kennel diet were found to vary from 241 to 318 mg. per 100 gm. of moist tissue (Table I). In selected normal dogs on the standard balanced diet the range was smaller, varying usually from about 226 to 270 mg. (Table II). In dogs maintained on the high fat diet for 20 days or longer these values were

reduced to 141 and 211 mg. respectively (Table IV). Thus there is a very definite relationship between the diet and the total phosphates in the liver. There are very few values for total phosphates in the livers of normal dogs to be found in the literature. Reed and his coworkers (19) in 1933 reported values for thirteen

TABLE I

*Phosphates in Livers of Normal Dogs on Regular Kennel Diet**

Dog No.	Hrs. fasted	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glycogen	Per cent water	
		Total	Acid-soluble	Phospholipids	Residual by difference	Fraction A (barium-soluble)	Alcohol-insoluble	Alcohol-soluble by difference	Fraction B (barium-insoluble)	Inorganic	Labile			Rest by difference
1	24	294	86			34.3	25.5	8.8	49.0	17.3	14.3	17.4	2.96	71.3
2	24		101			50.7	30.0	20.7	50.3	18.5	15.5	16.3	1.94	71.3
3	24	294	100	95	99	55.4	39.0	16.4	43.9	16.7	11.2	16.0	2.66	73.5
4	48	246	88	89	69	46.4	33.8	12.6	39.0	23.2	6.7	9.1	0.82	74.6
5	96	303	100	110	93	51.6	31.8	19.8	42.9	22.3	9.0	11.6	1.30	71.4
6	120	318	96	122	100	51.2	30.3	20.9	40.4	21.4	8.4	10.6	1.38	74.0
7	18	274	84	92	98	53.1	36.3	16.8	31.6	17.6	9.0	5.0	2.75	73.8
8	18	317	92	109	116	46.4	34.7	11.7	39.4	21.4	8.6	9.4	0.87	74.4
9	18	296	94	111	91	50.6	39.0	11.6	49.2	22.4	11.8	15.0	3.57	73.3
10	18	241	81	89	71					13.9	12.0		5.28	71.8
11	48	310	99	97	114	54.6	43.2	11.4	41.1	22.9	7.7	10.5	2.84	71.9
12	48		94			57.9	48.4	9.5	36.2	16.0	10.6	9.6	2.44	73.3
13			97							20.9	15.3		7.09	69.9
14		260	78	77	105					16.6	12.1		6.28	68.7
15	72		104			60.5	38.0	22.5	42.2	23.0	9.6	9.6	1.46	71.4
Average....		286.6	92.9	99	95.6	51.0	35.8	15.2	42.1	19.6	10.8	11.7	2.91	72.3

* Regular kennel diet: dog biscuits and mash made of ground horse meat, corn-meal, oatmeal, and bone ash. The dogs had access to this diet at all times.

normal dogs that ranged between 128 and 248 mg., the average being 197. Cullen, Wilkins, and Harrison (20) studied total phosphates in the livers of nineteen patients and found an average of 241 mg., the range being between 167 and 305 mg.

Phosphates in the liver are divided rather evenly into three

groups: acid-soluble, phospholipids, and residual phosphates, chiefly nucleic acid. This is in marked contrast to muscle, in which about two-thirds of the total is made up of acid-soluble

TABLE II
*Phosphates in Livers of Normal Dogs on Standard Mixed Diet**

Dog No.	Time, days	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glycogen	Per cent water	Per cent fatty acids
		Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol-insoluble	Alcohol-soluble by difference	Fraction B	Inorganic	Labile			
16	20	264	84	85	95	45.8	30.1	15.7	34.4	17.2	7.8	9.4	4.30	74.8
17	20	252	84	87	81	49.8	32.1	17.7	33.8	16.8	6.8	10.1	3.73	75.8
18	26	252	85	85	82	41.8	34.0	7.8	40.5	15.0	11.3	14.2	4.06	73.4
19	26	255	80	83	92	39.9	32.8	7.1	34.7	14.0	9.2	11.5	5.32	73.7
20	33	234	94	76	64	45.9	28.2	17.7	45.4	15.2	12.1	18.1	7.91	70.6
21	22	251	87	85	79	42.2	35.6	6.6	41.0	15.2	11.6	14.2	6.74	73.2
22	22	226	82	80	64	39.4	33.0	6.4	40.0	16.0	9.0	15.0	5.42	76.2
23	55	252	92	82	78	45.6	32.6	13.0	40.2	18.1	8.2	13.9	5.75	73.9
24	55	234	80	78	76	38.3	29.7	8.6	36.6	17.2	6.4	13.0	5.81	73.5
25	20	232	84	79	69	44.4	26.0	18.4	34.0	14.4	8.9	10.7	4.91	74.3
26	20	240	82	84	74	43.9	26.8	17.1	31.8	14.2	6.7	10.9	3.88	74.3
27	35	270	87	88	95	44.4	29.0	15.4	40.2	16.6	11.0	12.6	6.23	73.0
28	56	229	84	78	67	52.0	40.7	11.3	31.4	13.4	7.4	10.6	7.85	72.4
29	56	230	84	83	63	49.4	33.1	16.3	34.9	16.6	6.8	11.5	7.14	71.3
30	39	238	83	87	68	43.3	24.5	18.8	35.0	15.4	7.8	11.8	4.32	73.1
31	39	206	69	78	59	37.2	23.9	13.7	31.2	16.1	4.7	10.4	3.92	73.8
32	46	238	78	86	74	37.6	28.2	9.4	36.9	17.7	9.9	9.3	4.68	74.7
Average..		241.4	83.5	82.6	75.3	43.6	30.6	13.0	36.6	15.8	8.6	12.2	5.41	73.7

* Standard mixed diet: a daily weighed quantity of a mixture of 44 per cent meat, 44 per cent cracker meal, 8 per cent lard, 4 per cent bone ash, and a small quantity of tomatoes. Specimens of liver were obtained 18 hours after feeding, except in the cases of the last three dogs, when they were taken 24 hours after feeding.

phosphates. Very few studies have been carried out previously on the distribution of acid-soluble phosphates in the liver. In 1925 Cori and Goltz (21) studied the effect of insulin on inorganic phosphate and that split off by incubation with sodium bicarbon-

ate. They found a slight but distinct increase in inorganic phosphate, but no evidence of a glucosephosphate compound. Kay (22) also has made studies of the phosphates in liver, determining inorganic and total acid-soluble phosphates and obtaining the phosphoric ester content by difference. He found, in rats, that the acid-soluble organic phosphate slowly increases with increasing weight, and possibly also with increasing age. He

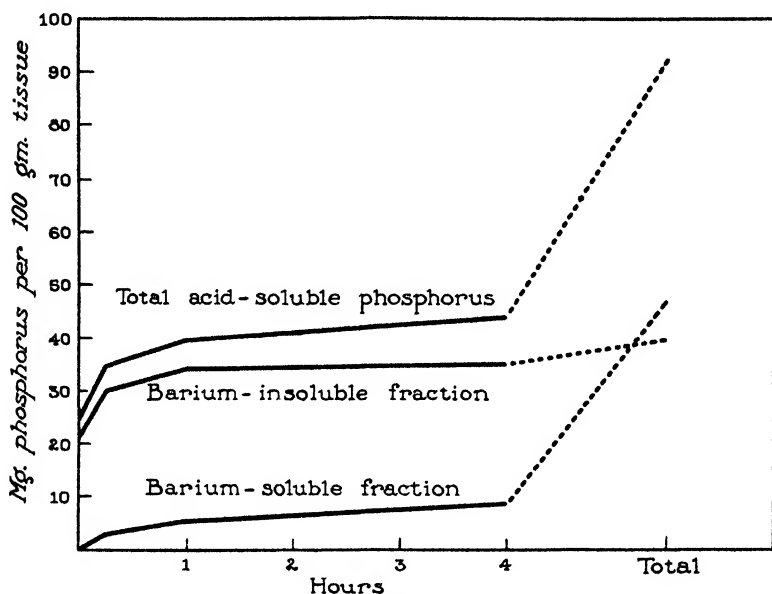


FIG. 1. The rate of hydrolysis of the acid-soluble phosphates of liver with normal sulfuric acid at 100°. The solid line represents inorganic phosphate split off; dotted line, organic phosphate not hydrolyzed in 4 hours.

found this fraction to diminish somewhat below normal in animals on a rachitogenic diet and to be increased back toward normal when antirachitic agents were added to the otherwise rachitogenic diets. In cases in which osteoporosis was produced in rats by a low calcium, high phosphorus diet, the phosphorus ester content of hepatic tissue was also diminished (23).

The scarcity of studies on the acid-soluble phosphates of liver is in marked contrast to the number which have been made on

muscle tissue, particularly during the last 10 years. These latter have yielded many interesting and important facts concerning the rôle phosphates play not only in the contraction of muscles, but also in the metabolism of carbohydrates. When one considers that as much glycogen can be stored in the liver alone as in the entire musculature, and that this quantity can show sudden and large increases and decreases, it seems that studies of the phosphates in hepatic tissue should also be worth while.

Our studies have included fractionation of acid-soluble phosphates as extensive as that previously employed for muscle tissue. The range of values for these fractions in normal dogs has been established (Tables I and II). As has been indicated, this range of values is fairly wide in the case of unselected dogs kept on the regular kennel diet, but, if selected dogs in excellent condition maintained on the standard balanced diet are used, the values are found to be very closely alike.

Experiments in hydrolysis have shown the presence of a compound in Fraction B sufficiently labile to yield inorganic phosphate on boiling for 15 minutes with normal acid, and other organic compounds which undergo practically complete hydrolysis of phosphate in an hour's time. On the other hand in Fraction A, both glycerophosphate and Compound X are very resistant to acid hydrolysis. While a few mg. of phosphate are split off in 15 minutes from them, only a minor part of the total phosphate is split off in 4 hours time. A much higher percentage of the acid-soluble phosphate compounds of the liver is relatively stable to acid hydrolysis than is true of muscle (Fig. 1).

Studies on administration of glucose yielded no evidence for a glucosephosphate compound during active synthesis of glycogen; nor has the formation of such a compound ever been directly demonstrated in the muscle of an intact animal during this process. That in the case of either or both tissues this is the result of a lack of sensitivity of analytical procedures is probable. The formation of such a compound appears to be the most logical explanation of the well established fact that both blood and urine show a marked decrease in inorganic phosphates during utilization of glucose.

The acid-soluble phosphates of liver are quite constant in amount and are not shifted easily by diet. The percentage composition is within the normal range for those dogs maintained on

TABLE III
Effects of Special Diets and Fasting on Phosphates in the Liver

Dog No.	Diet	Days	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glyco- gen	Per cent water	Per cent fatty acids	Weight of liver Weight of body	
			Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol- insoluble	Alcohol- soluble	Fraction B	Inorganic	Labile					Rest by differ- ence
31	Protein*	23	276	86	50	100	48.3	34.2	14.0	41.2	24.8	9.0	7.4	2.26	72.7		
32	"	42	260	84	92	84	37.9	28.4	9.5	42.6	20.9	7.8	13.9	5.90	70.6		
33	Carbohydrate†	27	257	86	91	80		38.0		36.0	17.6	9.6	8.8	10.87	70.7		
34	"	37	247	110	108	29	57.8	43.8	14.0	51.4	15.8	14.0	21.6	11.05	71.8		
35	"	35	236	84	73	79	36.1	24.6	11.5	45.2	15.0	17.8	12.4	7.48	72.0	1.7	4.4
36	"	48	250	97	84	69	48.7	27.3	21.4	47.8	17.5	16.8	13.5	7.12	73.2	2.5	4.6
37	Fat‡	9	245	83	80	82	46.3	27.7	18.6	31.2	16.2	8.0	7.0	1.72	77.6	3.1	2.9
38	"	22	275	75	107	93	43.2	25.4	17.8	32.0	24.6	4.6	2.8	2.30	71.1		
39	"	14	294	89	100	105	52.9	35.2	17.7	32.6	20.9	2.4	9.3	1.25	72.0	5.8	3.1
40	"	15	271	104	108	59	65.0	39.8	25.2	44.2	24.5	5.5	14.2	1.50	73.4	4.6	2.1
41	Fasting§	12	296	94	102	100	55.8	26.2	29.6	38.5	20.4	9.6	8.5	1.21	72.0	5.2	
42	" §	12	234	64	86	84	31.2	22.0	9.2	31.4	17.7	6.5	7.2	0.29	65.8	12.7	
43	"	15	322	82	113	127	42.4	31.9	10.5	41.2	25.4	6.2	9.6	2.10	73.6		
44	"	21	290	105	83	102	62.0	33.6	28.4	41.2	23.4	8.0	9.8	1.65	72.2		
45	"	21	261	71	100	90	36.0	30.9	5.1	37.4	18.2	12.5	6.7	0.48	67.6	10.1	3.4

* 50 gm. of horse meat per kilo of body weight per day.

† 5 gm. of horse meat and 11 gm. of crackers per kilo of body weight or 2 gm. of meat and 15 gm. of crackers.

‡ 3 gm. of horse meat and 8 gm. of lard (except for Dog 38 which received 5 gm. of meat and 5 gm. of lard).

§ Fasted after having been on the high fat diet for 12 days. Dogs 43 to 45 had been on the regular kennel diet prior to the fast.

the regular diet, the standard diet, and on the high protein, high carbohydrate, or even the high fat diet, if this last is given for less than 3 weeks, as well as for those dogs which have been fasted for periods up to 21 days (Table III). In the cases of two of the fasted dogs, in which the water was somewhat reduced, the acid-soluble phosphates were slightly below normal.

There are conditions, on the other hand, which will produce drastic changes in these phosphates. The simple administration of a high fat diet for 3 weeks or longer will do it (Table IV). The acid-soluble phosphates may decrease to half their normal value. This change is accompanied by big decreases in glycogen and water and a big increase in fat. All fractions of the acid-soluble phosphates participated in this decrease. The average value for inorganic phosphate in the group was about 60 per cent of the normal; the average values for both glycerophosphate and nucleotide phosphate were 50 per cent of normal, whereas those for the unknown barium-alcohol-soluble compound were about 35 per cent of the normal.

By taking specimens of liver by biopsy it has been possible to follow the phosphates in the liver of the same animal before and after changes in diet. Thus the rate at which the values for phosphates return to normal when a dog is shifted from a high fat diet back to a carbohydrate or standard diet has been studied (Table V). Restoration of normal values is much more rapid and much more easily obtained in such cases than is the production of low values when the fat diet is employed. The glycerophosphate and the unknown compound of Fraction A are the most rapidly restored. The water content increases, as does also the glycogen, and the fatty acids decrease. Distinct changes have been found in 3 days in spite of the lack of appetite following operation; a complete return to normal was found in from 8 to 10 days. It would appear that the acid-soluble phosphates, particularly α -glycerophosphate and Compound X, are at least indirectly involved in carbohydrate metabolism. They decrease slowly with a pronounced decrease in carbohydrate metabolism and increase rapidly when a carbohydrate diet is restored.

Another relationship of α -glycerophosphate to carbohydrate metabolism is shown in the recent work of Cori and Shine (24). They found that the addition of this compound caused a greater

TABLE IV
Effect of Prolonged Administration of High Fat Diet on Phosphates in the Liver

Dog No.	Days	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent fatty acids	Per cent water	Per cent glycogen	Weight of liver Weight of body
		Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol-soluble	Fraction B	Inorganic	Labile	Resistant by difference				
46*	30	180	47	71	62	20.0	16.2	23.9	14.6	3.8	5.5	43.8		0.12	5.50
47†	25	188	61	79	48	26.8	18.8	30.4	13.2	13.0	4.2	55.8	27.4	0.17	7.50
48†	30	141	45	63	33	19.2	15.0	19.9	9.3	8.1	2.5	35.6	52.2	0.09	4.15
49†	28	164	52	73	39	30.8	26.7	26.5	16.8	4.7	5.0	46.3	32.0	2.32	4.15
50†	29	194	49	76	69	28.8	23.5	25.1	14.0	8.0	3.1	51.2	32.3	0.55	5.20
51†‡	36	199	52	74	73	29.9	23.5	24.6	15.2	4.1	5.3	36.3	44.9	0.19	
52†‡	41	189	50	75	64	26.9	23.2	25.0	14.2	6.4	4.4	45.2	37.7	0.14	
53§	20	192	44	66	82	19.8	17.7	24.4	14.5	3.9	6.0	47.3	34.8	0.10	
54§	20	211	51	73	87	22.0	17.9	30.6	13.6	6.2	10.8	57.4	23.4	0.35	
55§	35	152	38	55	59	19.0	16.8	2.2	12.6	2.6	5.0	43.3	47.5	0.17	
56§	35	157	42	54	61	20.0	17.1	2.9	13.4	2.0	4.2	39.9	46.5	0.07	
57§	42	184	54	62	68	37.2	23.3	13.9	11.1	2.5	5.7	48.4	33.1	0.59	
Average		179.2	48.7	68.4	62.0	25.0	20.0	24.1	13.5	5.4	5.1	45.9	37.4	0.40	

* Received 2 gm. of horse meat and 6 gm. of lard per kilo daily.

† Received 3 gm. of meat and 6 gm. of lard.

‡ Received 10 gm. of lecithin daily in addition to the fat diet for the last 7 days.

§ Received 3 gm. of meat and 10 gm. of lard.

TABLE V
Effect of Shifting from High Fat Diet Back to Carbohydrate Diet on the Phosphates in the Liver

Dog No.	Diet	Days	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glycogen	Per cent water	Per cent fatty acids	Weight of liver Weight of body
			Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol-insoluble	Alcohol-soluble	Fraction B	Inorganic	Labile	Rest			
52	Fat	41	189	50	75	64	26.9	23.2	3.7	25.0	14.2	6.4	4.4	45.2	37.7	
53	Glucose	3	234	74	78	82	49.3	32.2	17.1	26.2	14.2	4.6	7.4	57.6	24.7	
	Fat	20	192	44	66	82	19.8	17.7	2.1	24.4	14.5	3.9	6.0	47.3	34.8	
54	Carbohydrate	8	292	100	85	107	57.6	36.4	21.2	38.8	18.6	11.0	9.2	73.6	5.1	4.1
	Fat	20	211	51	73	87	22.0	17.9	4.1	30.6	13.6	6.2	10.8	57.4	23.4	
56	Carbohydrate	10	279	101	81	97	56.6	32.2	24.4	41.7	19.0	12.2	10.5	72.1	3.8	3.7
	Fat	35	152	38	55	59	19.0	16.8	2.2	20.2	12.6	2.6	5.0	43.3	43.5	
58*	Glucose	1	167	48	63	56	18.0	18.0	0	27.6	12.5	6.3	8.8	48.5	33.7	
	Fat	37	216	77	75	64	41.8	25.1	16.7	33.4	17.8	4.9	10.7	53.4	27.1	
	Glucose	2	208	80	66	62	43.0	29.6	13.4	33.2	14.8	8.1	10.3	60.5	19.1	

* Because of the poor condition of this animal the diet was shifted from 3 gm. of meat and 10 gm. of lard per kilo to 30 gm. of meat and 10 gm. of lard 2 days before the first biopsy. The acid-soluble phosphates had become normal and no further change occurred, although the fatty acids were still decreasing.

increase in fermentable carbohydrate during a 3 hour incubation of hepatic tissue in oxygenated bicarbonate-Ringer's solution than that which occurred without the added substrate.

Of importance are the experiments in which specimens of liver were obtained at different times by biopsy of individual animals

TABLE VI

Biopsy Studies on Dogs Previously Maintained on Standard Diet

Dog No.	Diet	Days	Phosphorus, mg. per 100 gm. hepatic tissue											Per cent glycogen	Per cent water	Per cent fatty acids
			Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol-insoluble	Alcohol-soluble	Fraction B	Inorganic	Labile	Rest			
18	Standard	20	252	85	85	82	41.8	34.0	7.8	40.5	15.0	11.3	14.2	4.06	73.4	2.6
	"	14	258	87	84	87	52.6	38.2	14.4	33.0	16.8	6.8	9.4	3.71	74.5	2.5
19	"	20	252	80	83	89	39.9	32.8	7.1	34.7	14.0	9.2	11.5	5.32	73.7	2.2
	"	14	258	94	94	70	51.2	33.8	17.4	39.8	17.9	11.7	10.2	4.04	73.6	2.0
60	" *	52	286	94	92	100	59.7	43.7	16.0	32.6	14.6	9.1	8.9	4.44	71.4	
	"	54	278	98	96	84	53.1	36.1	17.0	44.9	18.8	11.8	14.3	4.54	73.6	
16	"	20	264	84	85	95	45.8	30.1	15.7	34.4	17.2	7.8	9.4	4.30	74.8	2.3
	Fat†	12	267	85	84	99	53.3	33.8	19.5	33.4	17.1	5.2	11.1	1.52	75.1	4.3
24	Standard	55	234	80	78	76	38.3	29.7	8.6	36.6	17.2	6.4	13.0	5.81	73.5	2.2
	Fat†	14	229	69	77	83	33.8	25.4	8.4	33.9	14.4	5.0	14.5	3.34	76.4	4.4
61	Standard	18	223	77	84	62	32.4	28.4	4.0	37.0	17.9	7.4	11.7	5.35	76.1	2.1
	Fat†	10	232	82	89	61	41.8	28.6	13.2	36.2	17.5	8.0	10.7	5.16	72.3	4.1
25	Standard	21	240	82	84	74	43.9	26.8	17.1	31.8	14.2	6.7	10.9	3.88	74.3	2.3
	Fat†	15	234	86	91	57	55.6	41.3	14.3	29.2	20.7	0	9.2	2.63	72.8	4.9
17	Standard	20	252	84	87	81	49.8	32.1	17.7	33.8	16.8	6.8	10.2	3.73	75.8	2.3
	Fat†	12	232	65	82	85	38.2	29.0	9.2	29.8	17.0	3.7	9.1	0.65	65.7	14.7
	Standard	28	258	91	91	76	47.6	33.2	14.4	38.8	18.0	9.4	11.4	5.29	75.4	2.8

* Received 10 gm. of glycine daily in addition to the standard diet.

† Fat diet consisted of 10 gm. of lard and 4 gm. of the basal standard diet per kilo per day.

maintained continuously in excellent condition on the standard diet (Table VI). Whether the intervals between biopsies were 2 weeks or 6 weeks, practically identical values for phosphate were obtained. This demonstrates the constancy in the chemical composition of the livers of normal animals on a constant diet

and also serves as a check on the reliability of the experimental data. This uniformity of composition is also apparent in the cases of the four dogs given the fat diet for 10 to 15 days, when the fatty acid content had not yet exceeded 5 per cent. In the case of Dog 17 which had 15 per cent fatty acids after this same interval, there was a small but distinct decrease in the acid-soluble phosphates, but this was readily restored to normal when the animal was again given the standard diet. It is apparent that fatty livers do not develop in different dogs at the same rate. This experiment suggests also that the decrease in phosphates associated with fatty livers is a gradual process and not one associated merely with the most extreme cases of fat deposition.

Many studies have been reported in the literature concerning the occurrence of, and changes in, phospholipids of hepatic tissue. As is shown in recent reviews by Sinclair (25), Bloor (26), Artom (27), and others, this is still a very controversial field. This is in part due to deficiencies in the analytical procedures used for the determination of phospholipids, and in part due to the inherent difficulty in interpreting changes in the percentage content of substances in a tissue which can fluctuate in size as much as the liver can. Its increase in size when it becomes very fatty is shown by comparisons of the ratio of liver weight to body weight. Thus in six dogs with fatty livers these ratios were between 4.15 and 7.50, whereas in eighteen dogs with normal livers, they varied from 1.8 to 3.3. Kaplan and Chaikoff (28) reported ratios in normal dogs from 1.9 to 2.8 per cent, and in depancreatized dogs with very fatty livers from 7.2 to 10.8 per cent. Higgins, Berkson, and Flock (29) have also shown that, in rats, the liver may show a 20 to 25 per cent increase in weight within 6 hours after a meal with subsequent return to normal size.

The phospholipid content of the livers of normal dogs on the regular kennel diet was found to vary from 77 to 122 mg., and in selected normal dogs on the standard diet from 76 to 88 mg. per 100 gm. of moist tissue. In fifteen dogs, some of which were maintained on a high protein diet, a high carbohydrate, or a high fat diet for 22 days or less, or which were fasted up to 21 days after being previously on the regular kennel diet or else fasted for 12 days, after being on the high fat diet for 12 days, the range of phospholipid of from 83 to 113 mg. was well within the normal

range. In the cases of the twelve dogs kept on the high fat diet until the fatty acids of the liver were between 23.4 to 52.2 per cent the phospholipids showed a definite decrease. However, the decrease was less than that which occurred simultaneously in the acid-soluble phosphates. Phospholipids do diminish in percentage composition in extremely fatty livers, but they do not decrease as much as the acid-soluble phosphates and they do not increase from their low level as rapidly as do the acid-soluble phosphates when the high fat diet is changed to a high carbohydrate or standard diet.

It is interesting to compare the quantity of phospholipid found with the quantity of fat. In normal livers these two substances occur in practically equivalent quantities. However, fat can be increased up to 16 times the normal quantity and in no case is this accompanied by a simultaneous increase in phospholipid. On the contrary, a decrease occurs. If the suggestion were true that one function of the liver in the utilization of fat was the formation of phospholipids from fat, we might expect an increase in phospholipids when the liver is supplied so abundantly with fat. Artom (30) more recently claims to have positive evidence concerning the formation of phospholipids in the liver. By feeding to dogs large quantities of horse fat, up to 30 gm. per kilo at a single meal, he found a 30 per cent increase in phospholipid in 5 hours, with a return to normal by the 10th hour. Our experiments differ from his in that livers were removed 18 hours after the dogs were fed. However, Artom's increase seems related to a temporary plethora of fat, and it is difficult to see why a continual plethora would not produce a similar result. With the exception of the extremely fatty livers in which phospholipids are decreased, we have found them to be remarkably constant regardless of the dietary condition of the animal, as did Mayer and Schaeffer (31) in 1913.

There is no need for detailed consideration of the third division of liver phosphates, the residual phosphates, which are comprised chiefly of nucleic acids. These values are obtained by difference and are subject to the experimental errors involved in three different determinations. It is apparent, however, that they do roughly parallel the acid-soluble phosphates and phospholipids in quantity in the normal dogs and that they do show a distinct decrease in the extremely fatty livers.

In considering the decreases which we have found in phosphates in extremely fatty livers, it is obvious that some of the decrease in percentage composition can be explained merely as the result of a dilution of hepatic tissue with fat. That this cannot explain entirely the decrease in acid-soluble phosphates is obvious, because they decrease more than do, for instance, the phospholipids. If the data are calculated in terms of dry tissue, large decreases are found in all the three major divisions of phosphates in the fatty livers as compared with the normal. This is because the water content in the fatty livers has been so markedly reduced, having been replaced by the fat. Histologic sections indicate that the increased fat has not become an integrated part of the cells but exists as distinct globules within them. Therefore it was considered desirable to calculate the data not merely on a dry basis,

TABLE VII

Average Values for Phosphate from Tables I, II, and IV, Calculated As Mg. per 100 Gm. of Water-, Fat-, and Glycogen-Free Tissue

Diet	Total	Acid-soluble	Phospholipids	Residual
Kennel.....	1315	426	454	439
Standard.....	1292	447	442	403
High fat.....	1099	299	420	380

but on a dry fat-free basis. As glycogen is the third big variable, changing from as high as 8 per cent in normal livers to as low as 0.10 per cent in extremely fatty livers, it has been grouped with the fat and water. Part of the data from Tables I, II, and IV has been calculated on a glycogen-, water-, and fat-free basis (Table VII). The average value of 3 per cent for fatty acids has been used in Table I, as determinations of fatty acid were not made when the data were collected. On this basis also the total phosphates are found to be distinctly lower in the extremely fatty livers. This decrease is found in the three main divisions but is much greater in the acid-soluble fraction.

SUMMARY

Procedures have been developed for the fractionation of the total phosphates of hepatic tissue into the three main divisions:

the acid-soluble, the phospholipid, and the residual phosphates. The procedure previously used for the fractionation of acid-soluble phosphates in skeletal muscle, based largely on the different solubilities of the barium salts of these compounds, has been modified for use with hepatic tissue.

Values have been determined for these phosphate compounds in fifteen normal dogs maintained on the regular kennel diet and in seventeen selected normal dogs maintained on the standard diet. The values for acid-soluble phosphates were similar in these two groups, but there was a narrower spread of values in the latter group where both the condition of the animal and the diet used were more constant.

High protein, high carbohydrate, and high fat diets, and even fasting, did not shift the phosphates in the liver outside the normal range in these fifteen dogs. Marked decreases did occur, however, with the high fat diet if it was continued long enough to produce extremely fatty livers. In twelve dogs in which this occurred, each type of phosphate compound decreased, but the greatest decrease was in the acid-soluble phosphates and the smallest was in the phospholipids. Of the acid-soluble phosphates, the glycerophosphoric acid and the extremely soluble unknown phosphate showed the biggest decrease.

The effect of shift in diet was best studied by means of biopsy of the livers of individual animals taken at different times. If the diet was not changed, the chemical composition of the liver remained remarkably constant; if the diet was shifted from one high in fat to one made up of carbohydrates the normal composition was rapidly restored. When the diet was shifted from the standard to a fat diet, livers of different dogs did not become fatty at the same rate. Even if the increase in fat was not extreme, however, small but definite decreases in acid-soluble phosphates did occur.

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EFFECT OF CERTAIN SUBSTANCES ON THE PHOSPHATE COMPOUNDS IN THE LIVER OF THE DOG

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That the values for different phosphate compounds in the liver of the dog are not readily shifted outside the normal range by dietary procedures has recently been shown by us (1). Only when a high fat diet was given for 3 weeks or longer, an interval sufficient for the production of an extremely fatty liver, were the phosphates decreased to decidedly abnormal values. The present studies were carried out to determine the effects of certain substances on the phosphates in the liver. Epinephrine, thyroxine, phlorhizin, and carbon tetrachloride, all of which affect such constituents of the liver as glycogen and fat, were studied. The effect of a lack of insulin on the phosphates in the livers of depancreatized dogs was also studied. A detailed description of the analytical procedures used as well as the values for phosphates in the livers of normal dogs are given in the preceding paper (1).

EXPERIMENTAL

Three dogs were given injections of epinephrine (Table I). This substance produces a breakdown in glycogen in both muscle and liver, and in muscle tissue this is accompanied by an increase in hexosemonophosphate (2). Specimens of liver were obtained by biopsy before and after injection. Amytal was used as the anesthetic.

Dog 1, which had been fasted for 5 days, was given epinephrine by intravenous injection at the rate of 0.05 mg. per kilo of body weight per hour for 1 hour and 15 minutes. A specimen of liver was taken before and after the injection, and also after a 4 hour recovery period. Dog 2, which had been given an injection of 17.3 gm. of glucose 45 minutes previously, was given 0.25 mg. of

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epinephrine intravenously over a period of 20 minutes. In this case, also, specimens were taken before and after the injection, but also after a period of recovery of $4\frac{1}{2}$ hours. In both these cases epinephrine produced a decrease in glycogen, but this was not followed by the expected increase during the period of recovery. Because this might have been indicative of an unsatisfactory physiologic condition, the control biopsy was omitted in the next experiment. Dog 3 received an injection of 0.25 mg. of epinephrine and biopsy was made 45 minutes and 5 hours later.

TABLE I
Effect of Injection of Epinephrine on Phosphates in Livers of Dogs

Dog No.	Time after injection of epinephrine		Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glycogen	Per cent water
			Total	Acid-soluble	Phospholipids	Residual by difference	Fraction A (barium-soluble)	Alcohol-insoluble	Alcohol-soluble by difference	Fraction B (barium-insoluble)	Inorganic	Labile	Rest by difference	
	hrs.	min.												
1	0	0	318	95.8	122	100	51.2	30.3	20.9	40.4	21.4	8.4	10.6	1.37
	1	15	297	101.8	118	77	50.0	30.7	19.3	48.5	24.8	7.6	16.1	0.22
	4	0	296	103.9	125	67	51.0	35.3	15.7	50.0	28.2	8.2	13.2	0.20
2	0	0	274	84.4	92.4	97.2	53.1	36.3	16.8	31.6	17.6	9.0	5.0	2.75
	1	0		74.9	79.1		41.5	29.2	12.3	35.0	17.5	6.2	11.3	1.06
	4	30	308	92.5	102.8	112.7	50.8	41.3	9.5	43.6	24.0	8.4	11.2	0.34
3	0	0												
	0	45	317	92	109	116	46.4	34.7	11.7	39.4	21.4	8.6	9.4	0.87
	5	0	304	86.3	110	108	52.5	41.8	10.7	31.9	18.4	4.3	13.1	1.64

There was an increase in glycogen during the period of recovery; however, there was no striking or consistent shift in any of the acid-soluble phosphates or in the phospholipids during the breakdown or subsequent increase of hepatic glycogen caused by the injection of epinephrine.

Four dogs (Nos. 4 to 7, inclusive, Table II) were given thyroxine subcutaneously for from 4 to 7 days, during which time they lost from 7.5 to 11.5 per cent of their weight. In the livers of Dogs 4, 5, and 6, which were maintained on the standard diet, the glycogen, water, and fatty acids were normal as were also the various phos-

TABLE II
Effect of Certain Chemicals and of Pancreatectomy on Phosphates in Livers of Dogs

Dog No.	Treatment	Diet	Days	Phosphorus, mg. per 100 gm. hepatic tissue										Per cent glycogen	Per cent water	Per cent fatty acids	Weight of liver
				Total	Acid-soluble	Phospholipids	Residual	Fraction A	Alcohol-insoluble	Alcohol-soluble	Fraction B	Inorganic	Labile	Rest by difference			
4	Thyroxine	S*	4	87	90	90	90	43.2	36.2	7.0	39.8	18.8	10.4	9.6	73.6	3.8	3.8
5	"	"	4	267	79	98	90	44.0	27.8	16.2	29.3	17.4	6.8	5.1	72.8	2.7	3.94
6	"	"	6	285	86	97	102	43.6	32.0	11.6	33.8	17.5	8.7	7.6	71.4	2.8	3.41
7	"	F†	7	248	64	112	72	32.6	25.0	7.6	32.5	18.0	6.2	9.4	61.4		
8	Phlorhizin	S	3	84	106			50.7	31.7	19.0	35.5	16.9	11.1	7.5	74.5	4.1	
9	"	"	3	88	91			48.6	33.2	15.4	33.7	21.3	6.4	6.0	73.4	4.0	
10	"	F	4	266	72	92	102	35.7	33.0	2.7	41.2	23.0	6.0	12.2	66.9	15.9	4.29
11	"	"	6	242	75	84	83	37.0	30.9	6.1	35.0	20.3	6.1	8.6	54.2	23.7	4.03
12	"	"	2	207	53	102	52	25.2	18.2	7.0	26.4	16.0	4.2	6.2	49.1		
13	Carbon tetrachloride	S	4	239	82	80	77	51.5	42.5	9.0	28.9	15.5	5.9	7.5	72.4	14.7	2.6
14	"	"	4	220	89	74	57					19.0	14.6		77.5	5.2	2.9
15	"	F†	4	202	72	72	58					17.3	11.4		74.7	9.1	2.1
16	"	"§	4	225	86	58	81	53.3	36.0	17.3	28.9	15.2	6.2	7.5	69.8	12.7	2.5
17	Pancreatectomy without insulin																
18	"	"	6	268	80			35.5	17.0	18.5	43.2	20.7	7.9	14.6	0.26		
19	"	"	5	202	60	66	76	33.1	20.7	12.4	29.2	17.4	5.2	5.6	54.8		
20	"	"	5	259	70	67	122	35.5	26.4	9.1	33.1	20.6	4.4	8.2	65.4	13.7	4.23
21	"	"	4	181											56.4	24.6	3.65
			5	218	73	75	69	38.4	25.6	12.8	29.0	16.7	6.0	6.3	60.6	18.5	4.96

* Standard diet.

† Fat diet.

‡ Fat diet used 1 day only.

§ Fat diet used 2 days.

phates. Only in the dog which received the high fat diet (Dog 7) before and during the experiment were the glycogen and water below normal. The acid-soluble phosphates in the case of this dog (No. 7) were the lowest of the group; however, this dog had received the high fat diet of 2 gm. of meat and 6 gm. of lard per kilo per day for a month, and the diet itself could account for these changes. In all of these four dogs the phospholipids were normal. Mild thyrotoxicosis of short duration appears, therefore, to have no specific effect on the phosphates in the liver. Schmidt (3) has reported that, in rabbits, the injection of thyroxine over a period of 10 days produced a decrease in the phospholipids in the liver.

Five dogs (Nos. 8 to 12, inclusive, Table II) received daily subcutaneous injections of 1 gm. of phlorhizin in oil. Dogs 8 and 9, which were fed the standard diet, were given three injections and lost 0 and 6.5 per cent of their weight, respectively. The values for glycogen of 0.2 and 0.3 per cent were decidedly low, but the fatty acids at 4 per cent were only slightly above normal. The values for water were normal as were also those for phosphates. Dogs 10 and 11, which were fed a high fat diet of 3 gm. of meat and 10 gm. of lard per kilo per day throughout the experiment, were given four and six injections, and lost 18.4 and 14.7 per cent of their weight, respectively. The values for glycogen were extremely low, the fatty acids were elevated to approximately 16 and 24 per cent, respectively, and the water contents were reduced to 67 and 54 per cent, respectively. The phospholipids were normal; the values for acid-soluble phosphate were the same as some of the low normals and were therefore inconclusive. Dog 12, which was given a high fat diet containing 2 gm. of meat and 6 gm. of lard per kilo for 10 days before and during the 3 day experiment, was given only two injections of phlorhizin. The value for glycogen was very low, and the water content was 49 per cent. The fatty acids, which were not determined, would obviously have been very high. The phospholipids were normal, but both the acid-soluble and residual phosphates were markedly reduced. That this was really a cumulative effect and not just the effect of the fat diet is suggested by the fact that never were such low values obtained when the fat diet was administered for only 2 weeks. 3 and 4 weeks administration of such a diet would be necessary to produce such decreases in the phosphates.

Four dogs (Nos. 13 to 16, inclusive, Table II) were subjected to anesthesia with carbon tetrachloride vapor for 1 hour on 4 consecutive days. The values for water content were normal and the fatty acids were increased to from 5 to 15 per cent, but the ratio of the weight of the liver to the weight of the body was well within the normal range. The total phosphates in three cases were somewhat low, but this was not reflected in the acid-soluble phosphates. In general it might be said that no definite early changes in the phosphates were produced by the administration of carbon tetrachloride. The results of longer administration of this drug have not been studied.

Five dogs (Nos. 17 to 21, inclusive, Table II) were depancreatized and then maintained with insulin until recovery from the operation was complete. The administration of insulin was then discontinued and the animals were killed 4 to 6 days later. Again the changes in phosphates seemed to follow the variations in the values for water and fatty acids. The water content decreased and the fatty acids increased during these intervals, but unless these changes were very large they were not accompanied by definite changes in the phosphates.

While the number of dogs used in these various experiments was not large, there appears to be no specific early effect on the phosphates in the liver produced by administration of the various substances studied. The relationship between the phosphates and the water and fat content of the liver is in agreement with the results obtained in a much larger series of animals used in the studies on the effects of different diets.

SUMMARY

Epinephrine produces no marked change in the acid-soluble phosphates in the liver of the dog, although it does produce an increase in the hexosemonophosphate of muscle.

Thyroxine, phlorhizin, and carbon tetrachloride, administered to dogs over intervals of a few days, did not produce extremely fatty livers or low values for phosphates unless used in conjunction with a fatty diet. In these dogs, as well as in the depancreatized dogs deprived of insulin, only when the liver became extremely fatty were the values for phosphates reduced below the normal range.

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EFFECT OF AUTOLYSIS ON THE PHOSPHATE COMPOUNDS IN THE LIVER OF THE DOG

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To determine the relative lability of the phosphate compounds of the liver to enzymatic action a study has been made of the effect of autolysis on the liver of the dog. Sevringhaus (1) in 1923 demonstrated that inorganic phosphoric acid increases during autolysis of the livers of dogs and that this accounts for most of the postmortem acidity in hepatic brei. Rona and his coworkers (2) also found that phosphoric acid and not lactic acid plays a major part in this acidity. They thought the origin of this must be nuclein and phosphatides. More recently, Teorell and Norberg (3) studied the effects of autolysis on cat liver for periods ranging from 3 hours to several hundred hours at temperatures of from 2-4°. They found that inorganic phosphate increased with diminishing speed chiefly at the expense of the other acid-soluble phosphates, and also, to a smaller extent, at the expense of the lipids. Their data show that the last named was a source of inorganic phosphate only when autolysis was allowed to continue for 20 hours or longer.

The present work was carried out for the purpose of securing more information concerning the nature of the early autolytic changes.

EXPERIMENTAL

Normal dogs which had been maintained on the regular kennel diet of dog biscuits and mash were fasted for 24 or 48 hours. The livers were removed after the animals were anesthetized with amytal. Part of the liver was frozen immediately with a mixture of carbon dioxide ice and alcohol; the remainder was placed intact in a dish covered with a moist towel. After definite intervals

TABLE I

Effect of Autolysis on Phosphates in Livers of Dogs

Dog No.	Autolysis, min.	Phosphorus, mg. per 100 gm. hepatic tissue									Per cent glycogen	Per cent water	
		Total	Acid-soluble	Phospholipids	Fraction A	Alcohol-insoluble	Alcohol-soluble by difference	Fraction B	Inorganic	Labile			Rest by difference
1	0	310	102	97	54.6	42.2	11.4	41.1	22.9	7.7	10.5	2.84	71.8
	5		106						23.8			2.86	72.4
	15		107		54.5	41.6	12.9	37.2	28.6	2.4	6.6	1.66	72.2
	60		107		63.0	41.3	21.7	46.3	41.0	0	5.3		70.6
	120		115		57.1	38.0	19.1	50.0	48.2	2.4	4.2	2.13	72.2
	240		106	102	46.2	39.2	7.0	52.5	50.2	1.9	0.2	1.42	70.8
2	0	241	81	89					13.9	12.3		5.28	71.8
	15		81						22.4	3.4		5.17	71.6
	60		84						33.8	2.2		5.22	70.2
	120		79						37.5	3.1		4.10	70.9
	240		86	95					45.8	2.3		3.65	70.0
3	0		94		57.9	48.4		36.2	16.0	10.6	9.6	2.44	73.8
	15		96		63.9	51.8		34.1	27.0	2.0	5.1	1.14	73.9
	60		99		56.4	41.2		41.5	33.0	4.0	4.5	0.98	74.2
	120		101		51.8	39.0		51.6	42.2	4.4	5.0	1.04	72.5
	240		96		43.8	36.6		55.2	48.4	1.8	5.0	0.68	72.6
4	0		97						20.9	15.3		7.09	69.9
	15		99						28.9	4.5		4.06	72.5
	60		112						41.8	5.1		5.92	69.6
	240		110						48.7	1.3		4.71	70.6
5	0	260	78	77					16.6	12.2		6.28	68.7
	15		82						25.8	3.4		5.82	68.2
	60		89	84					33.2	5.4		5.65	68.2
	240		83	80					44.2	1.0		5.90	67.5
6	0		103		60.5	38.0	22.5	42.2	23.0	9.6	9.6	1.46	71.4
	15		101		61.6	41.3	20.3	38.2	31.6	2.4	4.2	0.72	71.2
	240		107		46.4	34.4	12.0	61.5	50.5	2.9	8.1	0.40	70.8
7	0	294	100	95	65.0	39.0	26.0	33.5	16.7			2.66	73.5
	240	303	111	105	50.0	23.4	26.6	59.5	49.0	0.5	9.0	1.50	72.9
8	0	246	88	89	46.4	33.8	12.6	39.0	23.2	6.7	9.1	0.82	74.6
	240	247	102	97	38.0	25.4	12.6	57.6	52.5	0.6	3.7	0.44	73.3
9	0	303	100	110	51.6	31.8	19.8	42.9	22.3	9.0	11.6	1.30	71.4
	240	303	118	108	42.6	26.0	16.6	70.2	62.0	0.1	8.3	0.38	72.3

ranging from 5 minutes to 4 hours, and at room temperature, additional samples were removed and frozen to prevent further autolytic changes.

The frozen tissue was ground, weighed, extracted, and then analyzed by methods previously described (4). Determinations were made of total phosphates, water, and glycogen. The phosphates were studied by the usual methods of fractionation and hydrolysis, the results all being recorded in terms of phosphorus.

Results

Nine livers were autolyzed for 4 hours (Table I). Inorganic phosphate showed a large increase in all cases, most of this coming from the breakdown of the acid-soluble phosphates. The phosphate hydrolyzable in 15 minutes or the labile nucleotide phosphate disappeared. Very little of any of the organic phosphates was left in Fraction B, which is made up of the barium-insoluble compounds. In contrast to this was Fraction A, containing the barium-soluble compounds, in which there was a breakdown of only 15 to 25 per cent of the phosphates during the 4 hours. Thus this latter fraction was more resistant to autolysis, just as it was also more resistant to acid hydrolysis. When the original level of glycogen was 2.8 per cent or lower, it decreased from 44 to 73 per cent; when the level of glycogen was above 5 per cent at the beginning, it decreased only from 6 to 34 per cent. There were no significant changes in the water content.

In six of these studies samples of liver were taken at intermediate intervals of time. These showed that the initial increase in inorganic phosphate was rapid. 15 minutes of autolysis are sufficient to produce an increase of 10 mg., which comes from the breakdown of the labile nucleotide. In one case a specimen was taken just 5 minutes after removal of the liver, but neither the inorganic phosphate nor glycogen was changed during this short period of time. Following the breakdown of nucleotide, during the 1st hour of autolysis there was a subsequent increase in inorganic phosphate, at a diminished rate at the expense of the more stable esters. A further but still slower increase occurred during the next 3 hours, and this was due to the continued breakdown of these stable esters and in a few cases to the breakdown of the residual or nucleic acid fraction which is obtained by difference

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from the sum of the acid-soluble and phospholipid phosphorus and the total. There was no decrease in the phospholipids.

SUMMARY

Autolysis of hepatic tissue at room temperature for 15 minutes produces almost complete breakdown of the phosphate hydrolyzable in 15 minutes from the labile nucleotide to inorganic phosphate. During 4 hours of autolysis there is a further but slower increase in inorganic phosphate which is due to the breakdown of the more stable acid-soluble esters. Occasionally there is a further increase as a result of the breakdown of the residual or nucleic acid fraction.

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BLOOD PHOSPHOLIPID AS A TRANSPORT MECHANISM

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The belief that phospholipid is transported in the blood from the liver where it is formed to the other tissues of the body where it is burned has been almost universally accepted for nearly half a century. Nevertheless, the evidence upon which that idea is based is by no means decisive and, in a number of cases, is equally open to a different interpretation. In a review of the physiology of the phospholipids (1), this evidence was fairly completely discussed and therefore need not be cited here, except in so far as it has a direct bearing on the findings reported in this paper.

If all or even a significant portion of the fatty acids which are burned by an animal during fasting or on a high fat diet are transported in the blood in the form of phospholipid, then blood phospholipid must be undergoing a rather rapid turnover. This follows from the fact that the amount of fatty acid burned per day may be 50 to 75 times as great as the amount present at any time in the form of phospholipid in the blood stream. The measurement of the rate of turnover ought therefore to yield direct evidence as to whether or not blood phospholipid does actually serve as a mechanism for transporting fatty acids to the tissues where they are burned. Moreover, a comparison of the rates of turnover of the phospholipids in the plasma and the red blood cells ought to settle the still disputed point as to their relative participation in fatty acid transport.

Theoretically, it is possible to measure the rate of uptake of blood phospholipid by the metabolizing tissues by determining the concentration in the blood entering and leaving the organ and the rate of blood flow. Although it has been used with success by Nedswedsky and Alexandry (2) and recently by Boyd and Wilson (3), the method is subject to many complicating factors.

An independent and, in many respects, a more decisive method is to feed fatty acids which are permanently labeled in some way and to determine the rate at which these labeled fatty acids appear in the phospholipids. The difficulty has been in finding a suitable way of labeling the fatty acids. In 1929 some experiments were reported before the Thirteenth International Physiological Congress (4) which showed very definitely that the feeding of cod liver oil to dogs caused the average iodine number of the fatty acids of the plasma phospholipids to increase from 105 to 124 within 6 or 8 hours. There was no change whatsoever in the iodine number of the phospholipid fatty acids of the red blood cells. Further study of the effect of food fat on the degree of unsaturation of tissue phospholipids showed, however, that a rapid increase in iodine number could not be taken as a sign of a rapid turnover in the sense that the phospholipids were acting as intermediaries in fat metabolism. Accordingly, with Mr. Clair Smith, an extensive study was made of the comparative rates of increase and decrease of the iodine number of the plasma phospholipids of dogs when various fats were fed. However, the results obtained did not give a decisive answer to the question at issue.

In 1933 Artom (5) published the results of his study of the rôle of blood phospholipid as a transport mechanism in which he used iodized fatty acids. His data showed quite definitely that such entirely foreign fatty acids, when fed or injected intravenously, do enter into the phospholipids of the liver and the blood. The amounts of iodized fatty acids which Artom recovered in the phospholipids were, however, very small, especially when compared with the total amount of phospholipid fatty acids. It seems likely, as he suggested, that this is due to two factors, one the unnaturalness of the fatty acids used, and the other the splitting off of the iodine from the fatty acids. It was apparent, therefore, that there was still need for further study of the turnover of blood phospholipid by means of labeled fatty acids.

Recently it has been shown that elaidic acid, the stereoisomer of oleic acid, is well tolerated by rats, even when fed in very large amounts, and can be found in the phospholipids of the liver, muscles, and kidneys to the extent of about one-third of the fatty acids (6). There was good reason to believe, therefore, that

elaidic acid was highly suitable as a distinctive fatty acid for use in the study of the rate of phospholipid metabolism in blood and various other tissues. In this present paper are reported the results of the study of the rate and extent of uptake of elaidic acid by the phospholipids of the plasma and red blood cells of the cat.

EXPERIMENTAL

The method for the determination of elaidic acid has already been given in detail (6, 7). In brief, it makes use of the fact that the lead salt of elaidic acid, like those of palmitic and stearic acids, and unlike the lead salts of oleic and the other common unsaturated acids, is practically insoluble in cold alcohol. The fatty acids obtained on saponification of the phospholipids are dissolved in alcohol together with lead acetate and kept at 16° until the insoluble lead salts have settled out. These are centrifuged out and recrystallized once from alcohol. The free fatty acids are liberated, extracted with ether, and aliquots taken for the determination of the amount by the Bloor oxidative method and of the iodine number. The percentage of elaidic acid is calculated from the percentage and iodine number of the so called solid acids.

Elaidic acid was fed in the form of the mixed glyceride prepared by treating olive oil with N_2O_3 and crystallizing three or four times from cold acetone. Approximately 85 per cent of the acids consists of elaidic acid, the remainder presumably being the natural saturated fatty acids present in olive oil. This product, a white solid melting at about 42°, was fed by stomach tube in the form of an emulsion.¹ After varying times, as recorded in Table I, the cats were stunned by a blow on the head and bled into a flask containing 0.5 to 1 cc. of 30 per cent potassium oxalate, depending upon the size of the animal.

The blood was centrifuged at high speed for 30 minutes. The plasma (40 to 50 cc.) was aspirated into a 60 cc. separatory funnel, weighed, and then added dropwise to 400 cc. of 3:1 alcohol-ether. After removal of the residual plasma and the white blood cells,

¹ Quite good emulsions were obtained by dissolving about 300 mg. of liver lecithin in 20 to 30 gm. of melted elaidin and shaking up with 15 cc. of warm water.

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a weighed portion of the red cells was stirred up with 3:1 alcohol-ether in a mortar² and transferred to a flask with a total volume of about 400 cc. of alcohol-ether. The suspensions of plasma proteins and red blood cells in alcohol-ether were boiled for a few minutes on the steam bath and filtered. The proteins and cells were returned to the flasks, reextracted with about 150 to 200 cc. of alcohol-ether, and again filtered. The combined extracts were evaporated almost to dryness under reduced pressure with a stream of CO₂ to prevent violent ebullition, and the residue taken up in ethyl ether³ and transferred to a small flask. The ether solution was then evaporated almost to dryness and the lipid material was transferred to a 50 cc. centrifuge tube with petroleum ether. Further treatment was essentially as described elsewhere (7).

The data showing the percentage amount of the mixed phospholipid fatty acids of the plasma, the corrected percentage and iodine number of the solid acids, and the calculated values for the percentage of elaidic acid in the total phospholipid fatty acids of the plasma and red cells are shown in Table I. It perhaps needs to be emphasized that the separation of mixed fatty acids into the saturated (solid) and the unsaturated (liquid) acids on the basis of the differences in the solubilities of the lead salts in cold alcohol is by no means complete. Consequently, as will be observed, the solid acids always have a very appreciable iodine number and therefore an apparent elaidic acid content. There is no reason for thinking that the unsaturated acid in the solid acid fraction of the phospholipid fatty acids of control animals is really elaidic acid; rather, it is quite probable that most if not all of the unsaturation is due to lead oleate carried down with lead stearate and palmitate. In the five control cats, the apparent elaidic acid content of the phospholipid fatty acids ranged between 3.0 and 6.4 per cent for the plasma and between 3.4 and 6.2 per cent for the red blood cells. These values tend to be appreciably higher than the corresponding ones for the liver and muscle of rats which averaged 2.8 ± 0.5 and 2.3 ± 0.8 per cent, respectively.

² It was found that by rubbing up the red blood cells with successive portions of alcohol-ether the frequently troublesome clumping of the cells could be completely avoided.

³ The ethyl ether used was of good grade, free from peroxides.

On the other hand, they agree quite well with the values of 3.6 to 5.7 per cent for dog plasma as calculated from the data in Table III of a previous paper (7). These differences, though certainly significant, are of little importance to the problem under consideration. One must merely remember that a value of about 5 per cent elaidic acid is the base-line. In general, values below 10 per cent are not regarded as indicative of the presence of

TABLE I
Elaidic Acid Content of Blood Phospholipids

Cat No.	Absorption period	Plasma phospholipid fatty acids				Red cell phospholipid fatty acids		
		Amount per 100 gm. plasma	Solid acids		Elaidic acid	Solid acids		Elaidic acid
			Percent-age	I No.		Percent-age	I No.	
	hrs.	gm.			per cent			per cent
7	Control	0.063	33.4	8.0	3.0	35.1	8.8	3.4
8	"	0.106	44.1	11.3	5.5	36.0	13.1	5.2
9	"	0.186	41.4	14.0	6.4	34.5	16.2	6.2
10	"	0.105	40.5	11.2	5.0	39.9	10.2	4.5
11	"	0.145	39.8	10.2	4.5	39.4	10.7	4.7
1	6	0.116	40.7	13.3	6.0	25.6	9.2	2.6
6	8	0.115	45.9	35.3	18.0	33.3	0(?)	0(?)
13	8	0.116	51.2	38.8	22.1	39.2	10.6	4.6
3	12	0.081	49.9	42.9	23.8	31.5	8.5	3.0
5	12	0.187	52.0	48.2	27.9	36.7	7.4	3.0
4	15	0.109	45.4	47.0	23.7	32.4	9.8	3.5
12	18	0.104	52.7	40.2	23.6	38.5	11.6	5.0
14	48*	0.107	56.7	57.9	36.5	39.7	15.6	6.9
15	120†		54.5	44.2	26.8	41.7	16.7	7.7

* 20 gm. of elaidin twice a day, the last dose 12 hours before death.

† 15 to 20 gm. of elaidin twice a day. The last dose, given 9 hours before death, was vomited.

elaidic acid unless they fall in a regular sequence with higher values corresponding to a longer time period.

On comparing the values for the elaidic acid content in the controls with the corresponding values for the animals which were absorbing elaidin, two facts are immediately observed. One is that the phospholipids of the blood plasma contain very considerable amounts of elaidic acid within a few hours after its

ingestion. For example, elaidic acid makes up about 20 per cent of the phospholipid fatty acids after 8 hours. The other is that there is no evidence whatsoever that the phospholipids of the red blood cells contain any elaidic acid, with the exception of the two animals which had been fed elaidin for 2 and 5 days. Accordingly, the phospholipids of the plasma are transporting elaidic acid; those of the red cells are not.

On comparing the amounts of phospholipid fatty acid in the plasma of the control and elaidin-fed cats, it is evident that the variations are too great to give any indication as to whether or not there had been an increase as a result of the elaidin ingestion.

As the percentage of elaidic acid in the plasma phospholipid increases, the percentage of solid acids also increases, although the larger portion of the elaidic acid has taken the place of the completely saturated fatty acids, stearic and palmitic. To what extent the elaidic acid in the plasma phospholipids is present as a dielaidyl lecithin, for example, and to what extent it is present in conjunction with one of the natural fatty acids in a phospholipid molecule cannot, unfortunately, be calculated at present. Likewise, it is impossible to say whether all or only a part of the plasma phospholipid is engaged in fatty acid transport, although the fact that most of the values for elaidic acid fall between 24 and 28 per cent even 18 hours after ingestion of the elaidin and the maximum value reached was only 37 per cent may mean that only a part is so engaged. In a problem of this sort we are faced by the difficulty of not knowing to what extent the ingested fat and to what extent previously stored fat are being transported and burned at any one time. It seems reasonable to suppose that provided the rate of absorption equals or exceeds the rate of combustion of fat, then the ingested fat will be burned exclusively. The difficulty is to know how to attain and to maintain such a state. Also involved here is the question of the modification of ingested fat during passage through the intestinal wall, either by saturation or desaturation or by admixture with fat secreted into the intestine (8-10).

In two cats the non-phospholipid fatty acids of the blood were analyzed for their elaidic acid content. One animal, Cat 5, showed a very marked lipemia when killed 12 hours after the administration of elaidin. The plasma was found to contain 1670

mg. per cent of "fat" fatty acids, of which 59 per cent was elaidic acid. It will be recalled that the elaidin fed consisted of 85 per cent elaidic acid. Why the blood fat did not more closely approximate the composition of the ingested fat is not clear. It is certain that there was not sufficient cholesterol ester fatty acid (which necessarily was mixed with the glyceride fatty acids) to affect the results to such an extent. The other animal, Cat 14, showed only a slight lipemia 12 hours after the last of four doses of elaidin. The plasma "fat" fatty acids amounted to only 112 mg. per cent, 39 per cent being elaidic acid.

DISCUSSION

It has already been emphasized above that these experiments with elaidic acid show quite clearly that whereas the phospholipids in the blood plasma are engaged in fatty acid transport, the phospholipids of the red cells are not. These results agree, therefore, with our earlier experiments (4) cited above. On the other hand, Artom (5) and Artom and Peretti (11) recovered more iodized fatty acids in the phospholipids of the red cells than in those of the plasma. In their experiments, however, the amounts of the labeled fatty acids recovered in the phospholipids were very small in comparison with the total amount of phospholipid fatty acid. Thus, the maximum amounts of iodized fatty acids, in gm. per 100 gm. of tissue, were 0.022 for liver, 0.003 for plasma, and 0.009 for red blood cells; per 100 gm. of phospholipid fatty acids, the values become about 0.8, 2.0, and 3.3 per cent, respectively. It will be seen that their maximum value of 2 per cent of iodized fatty acids in the plasma phospholipid fatty acids is very small as compared with our maximum of 37 per cent. It is a question how much significance should be attached to the disagreement between Artom's results and our own in the case of the red blood cells. The lead salt method, because of the unavoidable loss due to the slight solubility of lead elaidate in cold alcohol, is incapable of detecting very small amounts of elaidic acid. Consequently one cannot dismiss the possibility that elaidic acid was actually present in the phospholipids of the red blood cells in amounts comparable to the iodized fatty acids found by Artom; that is, about 3 per cent or less.

Artom attached considerable significance to the fact that more

iodized fatty acid was present in the phospholipids of the red blood cell than in the plasma and pointed out that this was in line with the suggestion advanced some years ago by Bloor (12) to the effect that the red cells participated more actively in phospholipid transport than did the plasma. This suggestion was based on the observation that after feeding olive oil to dogs the amount of phospholipid increased more in the red cells than in the plasma. With one exception (13), subsequent investigations along the same line failed to confirm this observation (14-16). Obviously the absence of elaidic acid from the phospholipids of the red blood cells and its presence in large amounts in those of the plasma are completely in disagreement with the suggested rôle of the red cell in phospholipid transport.

Although the data for the percentage and iodine number of the liquid acids have not been given, they have been determined in all cases. The iodine numbers of the liquid acids in the red blood cells are of some interest in that they tend to be quite high, most of the values lying between 200 and 230. The question of the function of the highly unsaturated fatty acids is still unsettled, the various suggestions being that they are an intermediary stage in fatty acid catabolism, that they are involved in cellular oxidations as an oxygen transport mechanism, and that they are important to the structural function of the phospholipids. It is of interest then that the red blood cells, which do not have an active metabolism and therefore neither burn fatty acids nor consume oxygen, contain such highly unsaturated fatty acids. Unless this is a hold-over from the actively metabolizing nucleated stage, the presence of these highly unsaturated acids would support the idea that they are involved in the structural or membrane function of the phospholipids.

According to present day conceptions, the phospholipid of the blood which is engaged in fatty acid transport originates in the liver, where it is synthesized from neutral fat. The evidence upon which that idea is based is largely of a circumstantial nature, although some, it is true, is quite direct. In a recent paper (1) the author suggested that the metabolic phospholipid of the blood originates in the intestinal mucosa in which there is known to be an active phospholipid metabolism (17). In the meantime that possibility has gained considerable support. Süllmann and Wil-

brandt (18) have demonstrated decisively that during fat absorption there is an increased amount of phospholipid as well as neutral fat in the lymph flowing from the intestine of the rabbit. Comparable experiments by other investigators on dogs have never yielded decisive results, although some increases in the phospholipid content of the thoracic chyle have been observed (19, 20). If, therefore, there is a flow of phospholipid away from the intestine by way of the lymph, it is likely that there is an equal, if not a greater, flow by way of the portal circulation despite the failure to detect it (18, 21). In a recent paper Artom, using iodized fat, has confirmed the occurrence of a rapid phospholipid metabolism in the intestinal mucosa and, on the basis of marked changes in amount in contrast to the constancy observed by Sinclair (17), has also suggested that the phospholipid which increases in the blood and in the liver as well has its origin in the intestinal mucosa. Still other evidence which can be cited in favor of the intestinal origin of metabolic phospholipid is the existence of an active secretion and reabsorption of fat in the intestine during fasting (9). In conjunction with the experiments reported in this paper, the author has determined the elaidic acid content of the phospholipids of the liver and various other tissues. The intestinal mucosa, as is to be expected, shows the most prompt and the most extensive entrance of elaidic acid into phospholipid combination; next comes the blood plasma; and third the liver. Since blood phospholipid certainly takes up more elaidic acid than that of the liver, and appears to do so at a more rapid rate, it seems very likely that the blood phospholipid—at least that formed during fat absorption—does not originate in the liver. This study is being continued.

SUMMARY

When elaidin is fed to cats, elaidic acid is found in very considerable amounts in the phospholipids of the blood plasma within a few hours. If elaidin is fed repeatedly for several days, as high as 37 per cent of the fatty acids in the plasma phospholipid consists of elaidic acid. Plasma phospholipid definitely serves as a mechanism for the transport of fatty acids to the tissues where they are burned.

Since no elaidic acid could be detected in the phospholipids of

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the red blood cells (except for small amounts after several days of repeated feeding of elaidin), it is concluded that the red cells do not participate in phospholipid transport.

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DIMETHYLGLYCINE BUFFER

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To replace the borate buffer, which undergoes chemical reactions with a number of biologically important compounds, the veronal buffer has been recommended.¹ It covers the pH range 7.3 to 9. There is still a gap between this buffer and the alkaline range of the phosphate buffer which in part may be just covered by Sørensen's glycine buffer. Glycine, however, has the disadvantage of chemically reacting with many compounds containing carbonyl groups, especially quinones. This gap in the buffer series was strongly felt by the authors in recent research. It can be satisfactorily filled in by dimethylaminoacetic acid (dimethylglycine) which not only covers this gap more satisfactorily but also is chemically more indifferent.

This substance, in the form of its sodium salt, is prepared as follows: Half of a cold solution of 75 gm. of NaOH in 200 cc. of water is added to a solution of 80 gm. of chloroacetic acid in 100 cc. of water, the mixture being kept cooled during the neutralization. This solution is then poured into 180 gm. of a 35 per cent aqueous solution of dimethylamine and the mixture cooled in running water for 15 minutes. The other half of the sodium hydroxide solution is added and the mixture is allowed to stand overnight. It is then evaporated *in vacuo* completely to dryness and the residue is extracted with a liter of boiling 95 per cent alcohol by refluxing for 15 minutes. The alcohol is filtered off while hot, and evaporated on steam to about 200 cc., after which it is set on ice. The crystalline deposit is recrystallized from 300 cc. of 95 per cent alcohol to which 10 cc. of water have been added and this process repeated three to four times, until the product

¹ Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).

gives only a very slight test for chloride ion. After drying in a desiccator for 4 days there remains a perfectly white, light and fluffy product in a yield of 56 gm.

$C_4H_9O_2NNa$. Calculated. N 11.20, Na 18.39
Found. " 10.97, 11.01, " 18.18

The preparation should contain no glycine and can be tested for it by the ninhydrin reaction. To show the range of this buffer the following data are given. 2.50 gm. of sodium dimethylglycine are dissolved to a volume of 100 cc. in water. This was expected to

TABLE I
pH Values Obtained with Various Amounts of HCl

N HCl	pH	N HCl	pH
cc.		cc.	
0.2	10.58	1.1	9.60
0.3	10.42	1.2	9.50
0.4	10.28	1.3	9.39
0.5	10.16	1.4	9.28
0.6	10.05	1.5	9.17
0.7	9.96	1.6	9.05
0.8	9.87	1.7	8.85
0.9	9.79	1.8	8.60
1.0	9.70		

be a 0.2 M solution. When titrated again standardized HCl with methyl red the titer appeared to be 0.187 M.

When 10 cc. of this solution are mixed with varied amounts of HCl, expressed in Table I in terms of cc. of N HCl, and made up with water to a volume of 20 cc., the following pH values measured at 30°, are obtained. The reference pH is that of standard acetate taken, 4.62.

It may be mentioned that the error of the glass electrode with this buffer is very small. A solution giving pH 9.49 with the hydrogen electrode gives 9.45 with the glass electrode, another of 10.23 gave 10.12.

THE ULTRAVIOLET ABSORPTION SPECTRA OF THYROXINE, THYRONINE, TYROSINE, DIIODOTYROSINE, AND THYROGLOBULIN*

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A determination of the absorption spectra or the extinction at a given wave-length of a solution containing compounds of the thyroxine series suggests a simple rapid method of identification. The measurement of absorption spectra of individual compounds in water and acid solution is therefore desirable; such data are presented here (1).

The *thyroxine*, *thyronine*, *tyrosine*, and *diiodotyrosine* were purchased from Hoffmann-La Roche, Inc., of Basel, Switzerland. They were not purified further. The thyronine was purchased as crystals. The thyroxine came as a 0.001 M solution of pH 11.5.

Thyroglobulin was prepared by Ostwald's method from the thyroids of hogs.

Distilled water was used to make up the solutions. The NaOH and other reagents were of c.p. quality.

Solutions were kept at 0° until used. Absorption measurements were made at 22° ± 3°. Extinctions were measured with a Judd-Lewis sectorphotometer backed by a quartz spectrograph. A set of Scheibe absorption cells permitted concentrations to be held fixed (2). The experimental procedure and precautions have been described (2). All of the solutions of the proteins were supplied by Professor David Rapport of the Medical School, Tufts College, Boston. The Judd-Lewis apparatus (2) was lent by Professor George Shannon Forbes of Harvard.

The results are given in Fig. 1. The curves show no marked

* Contribution No. 370 from the Research Laboratory of Physical Chemistry, Massachusetts Institute of Technology.

differences in the absorption spectra sufficient to warrant identification of one or more of the compounds in the presence of the

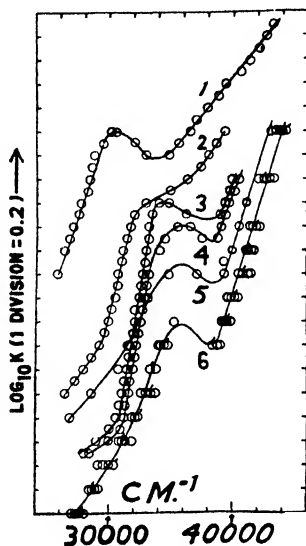


FIG. 1. Absorption spectra of thyroxine and related compounds. K $(1/cd)(\log_{10} [I_0/I])$; d in cm.

Curve No.	Compound	Concentration*	At initial point at the λ_{\max} . end of curves $\log K + \log c$ (c in moles per liter solution) equals
1	Thyroxine	0.0001 gm. per cc., pH 11.5	-1.2
2	Thyronine	0.001 M in 0.1 M NaOH	-1.4
3	Diiodotyrosine	0.001 " " 0.01 " " pH 9.0	-1.7
4	Tyrosine	0.001 " " 0.1 " " " 10.2	-1.7
5	Thyroglobulin	0.0001 gm. per cc. 0.001 M NaOH, pH 8.7	-1.6
6	"	0.0001 gm. per cc. 0.001 M NaOH, pH 8.7	-1.8

* Concentrations are as given by Professor Rapport.

others. Absorption measured in 3 and 1 cm. cells with a condensed hydrogen discharge as a source of continuous ultraviolet light likewise showed no sharp absorption bands. The Goertner

quartz spectrograph used in this part of the research had a dispersion of 10 inches from 15,000 to 50,000 cm^{-1} .

The points on Curve 5 are for thyroglobulin "denatured" by precipitation with acetone. The points on Curve 6 are for undenatured thyroglobulin, and for thyroglobulin denatured by grinding, by drying the ammonium sulfate precipitate, and by precipitation with methyl alcohol. Solutions of all these samples of thyroglobulin were kept in rubber stoppered glass bottles at 0° for 2 days before work on their absorption spectra could be started. Absorption measurements were made at $22^\circ \pm 2^\circ$ 4 hours after the solutions had been removed from 0° to a room at $22^\circ \pm 2^\circ$. Checks showed no changes in the absorption spectra of any of these solutions after standing at $22^\circ \pm 2^\circ$ for an additional 4 hours.

Within the limits of error, the curves show no differences between denatured and undenatured thyroglobulin, except when acetone was used as a denaturant. In the latter case (Curve 5) absorption was slightly greater and extended further into the visible region. This difference, however, may be due to the presence of some acetone still left in the thyroglobulin after "purification." Denaturing as carried out above, therefore, does not result in the elimination or formation of configurations within thyroglobulin which strongly influence the absorption spectra. Reprecipitation, with half saturated ammonium sulfate, of the denatured proteins which had been put into alkaline solution resulted in precipitates that were soluble at the isoelectric point; in other words, the denaturations in these particular cases were reversible.

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THE ERGOT ALKALOIDS

XI. ISOMERIC DIHYDROLYSERGIC ACIDS AND THE STRUCTURE OF LYSERGIC ACID

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(Received for publication, June 2, 1936)

Lysergic acid is a substance which we have shown to be formed on hydrolysis of all of the known ergot alkaloids and must therefore occur as such or in the form of an isomer in each of these substances. From our degradation studies¹ it has also been possible to arrive at a probable structure for lysergic acid, as given in the accompanying formula, and which appears to explain all of the known properties and reactions of this substance. Most of these have already been discussed, except in connection with a special type of transformation which these alkaloids undergo and which remains to be explained—namely, the isomerization of ergotamine to ergotaminine and the change of ergotoxine (probably a similar isomerization in spite of the recorded analytical difference of 1 mole of H₂O)² into ergotinine and of ergometrine into ergometrinine.

From our present knowledge it appears certain that the center of such isomerization lies in the lysergic acid molecule and not in the other cleavage products of these alkaloids. Thus, Smith and Timmis have recently described³ the isomer of ergometrine,

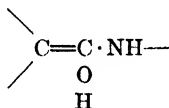
¹ Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **111**, 455 (1935); **113**, 767 (1936); *Science*, **83**, 38 (1936).

² From the analyses of ergotoxine and its salts the formula C₃₃H₄₁O₆N, has been derived, but it is not certain that retention of solvent may not have contributed to the analytical results. The observed greater acidity of ergotoxine as compared with the more basic properties of ergotinine may again be merely an apparent effect. This question is being carefully investigated by us. (Cf. Soltys, A., *Ber. chem. Ges.*, **65**, 553 (1932).)

³ Smith, S., and Timmis, G. M., *Nature*, **136**, 259 (1935).

ergometrinine, as strongly dextrorotatory and apparently of the same dextrorotatory group as ergotinine and ergotaminine. This alkaloid obviously results from isomerization of ergometrine in solution. The methyl alcoholic solution of the latter has been shown to mutarotate,⁴ becoming more dextrorotatory, which indicates partial transformation into such an isomer. There is no opportunity for such change to occur in the hydroxyisopropylamine portion of the molecule, thus leaving lysergic acid as the only center for such a change. Direct evidence for this we have been able to obtain in our hydrogenation studies.

In accordance with the proposed structural formula for lysergic acid, there are several possibilities which could be considered to explain its participation in the isomerization of the alkaloids. The asymmetric carbon atom, to which the carboxyl group is attached and which is joined in amide linkage in the alkaloids, could lose its asymmetry due to enolization



and since it is definitely known that other centers of asymmetry are present elsewhere in the alkaloid molecule, the result would be a mixture of epimers and not a racemic mixture. The individual epimers could be separated because of the difference in physical properties. This explanation has been excluded, however, since it has now been demonstrated that the double bond of lysergic acid is essential in the isomerization reaction.

It has been noted on a number of occasions that solutions of the alkaloids mutarotate, thus in the case of levorotatory ergotoxine by conversion into dextrorotatory ergotinine, and in that of levorotatory ergotamine because of change to dextrorotatory ergotaminine, and finally, as above stated, in the case of ergometrine (levorotatory in chloroform) because of change to the strongly dextrorotatory ergometrinine. Similarly, we have more recently found that lysergic acid methyl ester mutarotates on heating its methyl alcoholic solution, although the resulting transformation product could not be crystallized. On the other

⁴ Kleiderer, E. C., *J. Am. Chem. Soc.*, **57**, 2007 (1935).

hand, dihydrolysergic methyl ester did not change in rotation on boiling its methyl alcoholic solution. Likewise, dihydroergometrine could not be made to mutarotate. Thus, the double bond of lysergic acid must be essential for this change.

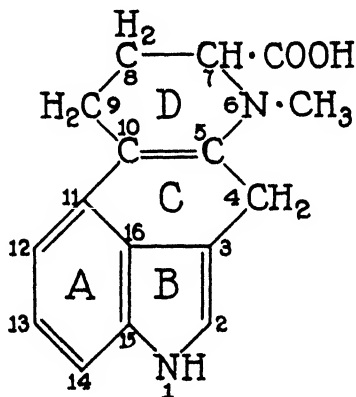
The subject has been investigated further from another angle. All of the alkaloids, whether of the levo or dextro series, which we have studied have been found to give the same lysergic acid on alkaline hydrolysis, which has in each case been confirmed by the preparation of the methyl ester. The question of its homogeneity will be discussed later. On the other hand, if the alkaloids were first hydrogenated (only in the case of dihydroergometrine was a crystalline alkaloid isolated) and then hydrolyzed, a striking result was obtained. The levorotatory alkaloids ergotoxine, ergotamine, and ergometrine gave in each case the same substance on alkaline hydrolysis, viz. α -dihydrolysergic acid ($[\alpha]_D = -110^\circ$). On the other hand, the dextrorotatory alkaloids, ergotinine, ergotaminine, and also the dextrorotatory cleavage product, ergine (lysergic acid amide), when first hydrogenated and then hydrolyzed gave an isomeric cleavage product which, for reasons given further on, we have designated as γ -dihydrolysergic acid ($[\alpha]_D = +30^\circ$).

Although in the operations with each series of substances the formation of still other isomeric dihydrolysergic acids is not excluded, none could be isolated. But the α derivative has appeared characteristic for the levo series and the γ derivative for the dextro series. The question remained as to whether such results could be conciliated with the structure for lysergic acid, which our degradation studies have led us to adopt. This will be seen to be possible in the later discussion.

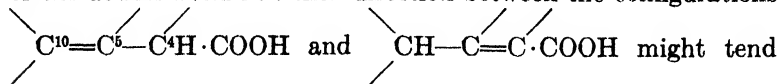
A further point has been the study of lysergic acid. Attempts to show that this acid as obtained from the various alkaloids is a mixture have convinced us of its homogeneity. Its properties appeared to remain constant on repeated recrystallization, even after recrystallization as the hydrochloride. The methyl ester, obtained in a yield up to 90 per cent, has also given no evidence of being a mixture. On the other hand, when lysergic acid is catalytically reduced, a definite mixture resulted from which both α - and γ -dihydrolysergic acids were isolated. Although it is remotely possible that lysergic acid may after all be a mixture of

acids belonging to each series of alkaloids, another interpretation appears much more probable; *viz.*, that new centers of asymmetry are produced by its hydrogenation with production of isomeric dihydrolysergic acids. This last interpretation we accept for the purposes of the argument.

Although evidence for the position of the carboxyl group is still lacking, this position appears to be restricted definitely to Ring C or Ring D. At present, it is assumed to be in Ring D, as given in the accompanying formula, and for convenience of discussion



we have assigned also numbers to the individual ring atoms. Position (4) for the carboxyl group, as presented in our previous paper,⁵ appears less likely because of the stability of dihydrolysergic acid which can be sublimed at 300° without appreciable decomposition. Again, with the carboxyl group at position (4) and the double bond between (4) and (5) or (5) and (10) (or (10) and (9)), as indicated by the ultraviolet absorption spectra,⁶ only one center of asymmetry could exist in either case, at (10) or at (4). Because of a more rigid configuration of the triad carbon atoms (4), (5), and (10), due to their occurrence in a ring (C) and not in an open chain, it might be expected that the shift of the double bond in either direction between the configurations

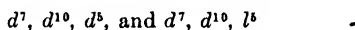


⁵ Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **113**, 771 (1936).

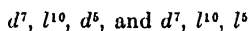
⁶ Jacobs, W. A., Craig, L. C., and Rothen, A., *Science*, **83**, 166 (1936).

to proceed with production of asymmetry at the carbon atom left by the double bond. Nevertheless, during the formation of lysergic acid under the conditions of alkaline hydrolysis, partial, if not total, racemization would be expected since each of the possible centers of asymmetry, carbon atoms (4), (5), and (10), can be involved during the shift of the double bond, and there would be no fixed center of asymmetry elsewhere in the molecule.

Position (7) for the carboxyl group appears more plausible. In this case, the configuration at carbon atom (7) could be maintained constant throughout and need play no rôle in the isomerism of the two series of alkaloids. The isomerism could be due (a) to epimerization on carbon atom (10) caused by shift of the double bond from between (4) and (5) to between (5) and (10) and back again to (4) and (5), or it could be caused (b) by a shift so that the double bond is fixed in one series between positions (4) and (5) and in the other between (5) and (10) (or (9) and (10)). If it is assumed that the first of these possibilities, (a), occurs with epimerization on carbon atom (10) with the double bond between (4) and (5), then the following isomers would be expected on hydrogenation. With the fixed configuration on carbon atom (7) of d^7 throughout these would be in the case of one series of alkaloids



and in the case of the other series



Thus, opportunity would be given for the production of different dihydrolysergic acids from each series of alkaloids.

If such an explanation for the isomerism of the alkaloids is accepted, the double bond in the case of lysergic acid, however, must be assumed to occupy rather the position between carbon atoms (5) and (10), perhaps because of the polar character of the free carboxyl group at (7). This would permit the simultaneous formation from this acid of two isomeric dihydrolysergic acids (α and γ) such as, for example, d^7, d^{10}, d^5 and d^7, l^{10}, l^5 , which, as above noted, are individually characteristic as products of each series of alkaloids. If the double bond were between carbon atoms (4) and (5) in lysergic acid, with fixed configuration at (10), then the conditions would be identical with what has been assumed

in the case of the alkaloids, and α - and γ -dihydrolysergic acid should not be simultaneously produced from it. Even if the double bond of lysergic acid were assumed to have a degree of lability between positions (5), (10) and (10), (9) with epimerization at (5), then all of the above four isomers would be simultaneously expected, thus giving opportunity for the formation of those encountered individually in the case of the alkaloids. If possibility (b), i.e. different positions (4), (5) and (5), (10) (or (10), (9)) for the double bond in each series of alkaloids, is assumed, a more involved situation would arise. Although possible, the chance for different substances preponderating as products of hydrogenation in each series of alkaloids would appear less likely than in the alternative scheme (a), and it is not clear that the divergent behavior of lysergic acid could be so well explained.

A final point has been the experience with the reduction of the isomeric dihydrolysergic methyl esters to the dihydrolysergols. In our previous work on the reductive cleavage of ergotinine and ergotamine with sodium and butyl alcohol,⁷ two isomeric alcohols, α - and β -dihydrolysergol, were obtained. Likewise, on similar treatment of lysergic methyl ester the same substances resulted. It was therefore of interest to attempt to correlate these dihydro alcohols with the two dihydrolysergic acids resulting from the hydrogenation and hydrolysis of the two series of alkaloids.

The above α -dihydrolysergic acid (from ergotoxine) has been found to be identical with the previously described dihydrolysergic acid obtained by reduction of lysergic acid with sodium in amyl alcohol.⁸ This was confirmed by the comparison of the methyl esters from both sources. If α -dihydrolysergic methyl ester is reduced with sodium in butyl alcohol, the resulting substance proved to be the alcohol, α -dihydrolysergol. On the other hand, when the methyl ester of γ -dihydrolysergic acid (from the dextro-rotatory alkaloids) was similarly reduced, an alcohol was also obtained which proved, however, to be different from the previously described β -dihydrolysergol obtained as a product of the reductive cleavage of ergotinine. This substance has therefore been called γ -dihydrolysergol and its precursor, γ -dihydrolysergic acid. The

⁷ Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **108**, 595 (1935); *Science*, **81**, 256 (1935).

⁸ Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **106**, 393 (1934).

previously mentioned β -dihydrolysergol obtained from the alkaloids was not, however, encountered in the reduction of the above α or γ esters. It is possible that this alcohol has its origin in epimerization on carbon atom (7), while the lysergic acid is still conjugated in the alkaloid. However, the exact relationship of these isomeric alcohols will require further investigation.

In the foregoing, the attempt has been made to reconcile the observed facts with the structural formula for lysergic acid, which has been deduced. This has been found to be possible, but at the same time such a formula is advanced with proper reservation because of the general nature of the evidence which is available. Attempts to synthesize a substance of the assumed structure for lysergic acid are now in progress.

EXPERIMENTAL

Catalytic Reduction of Ergotinine—0.2 gm. of ergotinine, $[\alpha]_D^{25} = +394^\circ$ ($c = 0.5$ in chloroform), was treated with 0.05 gm. of Adams and Shriner's catalyst and 2 cc. of glacial acetic acid. After shaking for 1 hour in hydrogen under about $2\frac{1}{2}$ atmospheres pressure, the rate of absorption of hydrogen had become much slower. A brilliant fluorescent purple-blue color developed immediately when the reduction started. The reduction was interrupted at the end of 3 hours when 1.8 moles of hydrogen had been absorbed.

The catalyst was filtered off and the black-colored filtrate was evaporated to dryness on the steam bath under reduced pressure. It was found to contain colloidal platinum which was removed by treating the ethyl alcoholic solution with bone-black. The still dark colored filtrate was evaporated to dryness again. All attempts to obtain the hydrogenated alkaloid in a crystalline form failed.

Hydrolysis of Hydrogenated Ergotinine. γ -Dihydrolysergic Acid—The crude amorphous product from above was dissolved in a solution containing 2 cc. of methyl alcohol, 2 cc. of water, and 0.56 gm. of potassium hydroxide. The mixture was refluxed in an atmosphere of hydrogen for 2 hours, and then diluted with an equal volume of water. After filtering from a slight amount of tarry material and removal of methyl alcohol by evaporation under reduced pressure, the solution was made slightly acid to Congo

red with sulfuric acid. The solution was then made alkaline with sodium carbonate and evaporated to dryness under reduced pressure. The solid residue was extracted with hot ethyl alcohol and the extract was evaporated to dryness. The residue was dissolved in 5 cc. of water and the solution was made slightly acid to Congo red with sulfuric acid. Ammonia was then added and the solution was concentrated over a free flame until crystalline material began to separate. The volume of solution at this point was approximately 2 cc. After cooling in ice for several hours, the crystals were collected with a little water. The yield was 35 mg. The material was further purified by recrystallization with bone-black from water. It is dextrorotatory. $[\alpha]_D^{25} = +32^\circ$ ($c = 0.22$ in pyridine). It does not show a sharp melting point, but darkens rapidly at 300° and decomposes at 330° when the temperature is raised rather rapidly.

$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 71.40, H 6.79

γ -Dihydrolysergic Acid Methyl Ester—70 mg. of γ -dihydrolysergic acid were treated with 5 cc. of absolute methyl alcohol and saturated with dry hydrogen chloride. After standing at room temperature for 1 hour, the solvent was evaporated under reduced pressure. The residue was treated with 10 cc. of ether and cooled in ice. Excess cold ammonia was added with good chilling and the mixture was shaken until all was in solution. The ether layer was dried over anhydrous potassium carbonate and then evaporated to dryness. The viscous residue could not be made to crystallize from any solvent. This residue was finally used for reduction to the alcohol as follows.

Reduction of γ -Dihydrolysergic Acid Methyl Ester. *γ -Dihydrolysergol*—65 mg. of the above amorphous ester were dissolved in 4 cc. of anhydrous butyl alcohol. The solution was boiled and 0.2 gm. of sodium was added. The mixture was at once vigorously shaken to emulsify the sodium and was maintained at the boiling temperature until all had dissolved. The solution was diluted with water and evaporated under reduced pressure until all butyl alcohol had been removed. The solid material which separated could not be extracted with ether. The mixture was acidified with a slight excess of sulfuric acid, and then neutralized with a slight

excess of sodium carbonate. After concentration of the solution to dryness under reduced pressure, the residue was extracted with hot ethyl alcohol. The alcoholic extract on evaporation left a residue. On digestion with 2 cc. of hot water, apparently crystalline material remained, which was collected with water. It weighed 30 mg. On recrystallization from methyl alcohol imperfectly formed tables or rhombs resulted. The material darkened at 234° and melted with decomposition at 255° depending somewhat on the rate of heating. It was dextrorotatory, $[\alpha]_D^{25} = +33^{\circ}$ ($c = 0.24$ in pyridine).

$C_{16}H_{20}ON_2$. Calculated, C 74.96, H 7.87; found, C 74.95, H 7.59

Catalytic Reduction of Ergotoxine—0.1 gm. of ergotoxine ethane-sulfonate (from Burroughs Wellcome and Company) in 2 cc. of glacial acetic acid was shaken with 50 mg. of Adams and Shriner's catalyst and hydrogen under an excess pressure of $1\frac{1}{2}$ atmospheres. After 2.5 hours, approximately 3 moles of hydrogen had been absorbed and the reduction had become rather slow. The catalyst was filtered off and the filtrate was evaporated to dryness under reduced pressure. The residue was treated with ethyl alcohol and again evaporated to dryness. The residue was treated with 5 cc. of water and a slight excess of ammonia. The amorphous alkaloidal precipitate which remained was filtered off. It weighed 70 mg. and could not be made to crystallize from any solvent.

Hydrolysis of Hydrogenated Ergotoxine. α -Dihydrolysergic Acid—The above amorphous residue was treated with a solution containing 1 cc. of water, 1 cc. of methyl alcohol, and 0.28 gm. of potassium hydroxide. After the material was refluxed for 1.5 hours in an atmosphere of hydrogen, the methyl alcohol was partially removed by evaporation under reduced pressure. The remaining solution was diluted with water and made barely acid to Congo red with sulfuric acid. This was followed by addition of ammonia in slight excess and upon concentration over a free flame to about 2 cc. when crystallization occurred. After cooling, 20 mg. of leaflets were collected with water. After recrystallization with bone-black from water, it darkened rapidly at 300° and finally decomposed at 330° , depending somewhat on the rate of

heating. It was levorotatory, $[\alpha]_D^{25} = -89^\circ$ ($c = 0.09$ in pyridine).

$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 71.17, H 6.72

This acid, which we shall call α -dihydrolysergic acid, was indistinguishable in properties from the dihydrolysergic acid previously described as a reduction product of lysergic acid.⁸ This was confirmed by the preparation of the following methyl ester which was prepared as in the case of the γ ester. After recrystallization from benzene, it melted at 182° after preliminary softening at 180° . A mixed melting point with the dihydrolysergic methyl ester previously reported showed no depression. It is levorotatory. $[\alpha]_D^{25} = -52^\circ$ ($c = 0.23$ in methyl alcohol).

Reduction of α -Dihydrolysergic Methyl Ester. α -Dihydrolysergol—50 mg. of the ester were reduced in 4 cc. of butyl alcohol with 0.2 gm. of sodium, as in the case of the γ isomer. The reaction mixture was worked up in identical manner. 20 mg. of crystalline material, which was sparingly soluble in water, were finally obtained. After recrystallization from methyl alcohol the material melted at 279 – 280° , depending somewhat on the rate of heating. A mixed melting point with α -dihydrolysergol⁷ showed no depression. Its identity was confirmed by its general properties and rotation, $[\alpha]_D^{25} = -86^\circ$ ($c = 0.27$ in pyridine).

$C_{16}H_{20}ON_2$. Calculated, C 74.96, H 7.87; found, C 74.90, H 8.16

Catalytic Reduction of Ergotamine and Hydrolysis—Ergotamine tartrate (Sandoz Chemical Works) was treated with sodium carbonate solution and the base was extracted with hot chloroform. The chloroform residue was recrystallized from dilute acetone. $[\alpha]_D^{25} = -140^\circ$ ($c = 0.43$ in chloroform). This material was used for the reduction. 0.1 gm. was dissolved in 2 cc. of glacial acetic acid and shaken with hydrogen and 25 mg. of Adams and Shriner's catalyst. When reduction began, a characteristic brilliant bluish purple fluorescence developed. After 2 hours, 1.6 moles of hydrogen had been absorbed and the absorption had become slow. The reduction products were hydrolyzed exactly as described in the case of ergotoxine. Upon recrystallization of the resulting dihydrolysergic acid, 18 mg. of the characteristic leaflets

of the α form were obtained. $[\alpha]_D^{25} = -115^\circ$ ($c = 0.235$ in pyridine).

$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 71.10, H 6.77

Catalytic Reduction of Ergotaminine and Hydrolysis—0.23 gm. of crystalline ergotaminine was dissolved in 10 cc. of methyl alcohol and refluxed in an atmosphere of hydrogen for 2 hours. The solution was cooled and the crystalline material collected. It weighed 0.07 gm. $[\alpha]_D^{25} = +382^\circ$ ($c = 0.23$ in chloroform). When the mother liquor was further refluxed, another crop of ergotaminine could be obtained.

0.1 gm. of ergotaminine was reduced in 2 cc. of glacial acetic acid with 25 mg. of catalyst. In 1.5 hours, 1.67 moles of hydrogen had been absorbed. The resulting products were treated exactly as described under the reduction and hydrolysis of ergotinine. Upon recrystallization of the dihydrolysergic acid, 12 mg. of the characteristic polygonal crystals of γ -dihydrolysergic acid were obtained. $[\alpha]_D^{25} = +32^\circ$ ($c = 0.22$ in pyridine).

$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 71.17, H 6.63

Catalytic Reduction of Ergine and Hydrolysis—0.1 gm. of ergine, $[\alpha]_D^{25} = +332^\circ$ ($c = 0.28$ in methyl alcohol), which was prepared from ergotinine according to the direction of Smith and Timmis,⁹ was hydrogenated in 2 cc. of glacial acetic acid with 25 mg. of catalyst. 1.6 moles of hydrogen were absorbed in 40 minutes, and the reduction became slow. The reduction products were hydrolyzed as described under ergotinine. 20 mg. of dihydrolysergic acid were obtained, which showed the characteristic polygonal crystalline form of γ -dihydrolysergic acid. $[\alpha]_D^{25} = +29^\circ$ ($c = 0.28$ in pyridine).

$C_{16}H_{18}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 70.96, H 6.99

Catalytic Hydrogenation of Lysergic Acid—The reduction of this substance has been reported.¹ It is described again below, since this procedure has yielded two isomeric dihydrolysergic acids.

0.4 gm. of lysergic acid was hydrogenated in 3 cc. of glacial acetic acid with 25 mg. of catalyst. The hydrogenation was in-

⁹ Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 763 (1932).

interrupted when 1.5 moles of hydrogen had been absorbed. The residue after removal of solvent was dissolved in 35 cc. of boiling water, bone-black was added, and then the mixture was quickly filtered while hot before crystallization began. The filtrate on cooling yielded 0.14 gm. of broad leaves which the rotation showed to be α -dihydrolysergic acid. $[\alpha]_D^{25} = -106^\circ$ ($c = 0.255$ in pyridine). This was confirmed by its conversion into the ester, as previously reported.

The mother liquor was concentrated until crystals appeared. After cooling, the somewhat colored crystalline material was collected and weighed 90 mg. It was recrystallized with bone-black, and the characteristic crystalline form of γ -dihydrolysergic acid was obtained. The rotation was slightly low for this compound. $[\alpha]_D^{25} = +16^\circ$ ($c = 0.235$ in pyridine). For further identification it was converted into the methyl ester which was in turn reduced to the corresponding γ -dihydrolysergol. The latter melted at 253° . $[\alpha]_D^{25} = +33^\circ$ ($c = 0.27$ in pyridine).

Mutarotation of Lysergic Acid Methyl Ester—Lysergic acid, $[\alpha]_D^{25} = +30^\circ$ ($c = 0.40$ in pyridine), was esterified, as previously reported, with diazomethane. It was found that upon recrystallization of this ester, the rotation varied somewhat. A solution of a sample which showed a rotation, $[\alpha]_D^{25} = +85^\circ$ ($c = 0.305$ in methyl alcohol), was heated in a sealed tube at the temperature of boiling methyl alcohol for 0.5 hour. The solution became slightly colored and the rotation increased to $[\alpha]_D^{25} = +118^\circ$ ($c = 0.305$ in methyl alcohol). The solution on heating an additional 2 hours became still darker in color, but the rotation remained constant at $[\alpha]_D^{25} = +118^\circ$. An attempt to isolate the products after the mutarotation of the ester yielded crystalline material which, however, showed a rotation approximately that of the original ester, *viz.* $[\alpha]_D = +80^\circ$ to $+90^\circ$. A large fraction could not be made to crystallize.

A solution of α -dihydrolysergic methyl ester showed no change in rotation after heating in boiling methyl alcohol for 1 hour.

DETERMINATION OF CHLORIDES IN BIOLOGICAL MATERIALS

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(Received for publication, May 20, 1936)

The methods used in determining the chloride content of blood, urine, and tissue all involve precipitation of the chloride with an excess of standard silver nitrate and titration of the excess precipitant with standard ammonium or potassium thiocyanate, with ferric alum as indicator. These methods are reasonably accurate and reliable but rather long and tedious. They also introduce several sources of possible error.

With the idea in view of shortening the time required and simplifying the procedure generally, a study was begun on the methods which had been used and others which might possibly be applied to the problem at hand. From the outset all gravimetric methods were ruled out, except as controls. They were considered to be too long and tedious for ordinary work on chlorides.

EXPERIMENTAL

The silver nitrate-thiocyanate procedures had already been brought down to a fair state of perfection, but still did not meet the author's requirements; so they were ruled out almost at the beginning of the experiment. After an exhaustive study of the literature Fajans' (1) technique, in which is used direct titration with standard silver nitrate and in which dichlorofluorescein is used as indicator, seemed to offer the only possibility worthy of further consideration.

A preliminary study of this procedure on known sodium chloride samples in acid, alkaline, and neutral solution brought out several interesting facts. (1) Digestion of sodium chloride on a boiling water bath for only 10 minutes in 2 N nitric acid caused a loss of

chlorides. (2) Titration in acid solution gave no end-point. (3) Titration in alkaline solution gave a gradually changing end-point which could not be read. (4) The procedure, when employed in neutral solution, was absolutely trustworthy.

The next point considered was the production of a satisfactory tissue digest. The requirements of this digest were: (1) it must be clear and sufficiently light in color to permit the reading of an end-point, and (2) it must be neutral in reaction. It was found, just as in the preliminary experiment, that digestion in any strength of nitric acid caused a grave loss of chlorides, although it gave a satisfactory light yellow-colored solution. Tissues digested in from 2 to 10 *N* potassium hydroxide gave solutions which were clear but highly colored. This color ranged from an orange to almost a black, depending on the amount of blood present in the tissue being analyzed. However, it was found that if this potassium hydroxide-tissue digest was made acid with nitric acid and then redigested, it cleared up to a light yellow color without any loss of chlorides.

The next step was to neutralize the solution to pH 7.0 to 7.5. This required an indicator that would accurately indicate this range and whose own color would not interfere with the reading of the end-point in the subsequent titration. A great number of indicators were tried, but all were found unsuited for various reasons. At the suggestion of Dr. A. M. Lands the B.D.H. universal indicator¹ was used and found to be quite satisfactory. At pH 7.0 to 7.5 its color is a greenish yellow which does not obscure the color change of the dichlorofluorescein in the subsequent titration. Its color range is from pH 3.0 to 11.0 as follows: up to pH 3.0, red; 4.0, deeper red; 5.0, orange-red; 5.5, orange; 6.0, orange-yellow; 6.5, yellow; 7.0 to 7.5, greenish yellow; 8.0, green; 8.5, bluish green; 9.0, greenish blue; 9.5, blue; 10.0, violet; 10.5, reddish violet; 11.0, deeper reddish violet.

The standard solution of silver nitrate used was prepared by dissolving 29.061 gm. of chemically pure silver nitrate in sufficient distilled water to make 1 liter. This gave a standard solution in which 1 cc. was equivalent to 10 mg. of sodium chloride.

¹ The B. D. H. universal indicator is prepared by The British Drug Houses, Ltd., and may be obtained in this country from Eimer and Amend, New York.

The actual method for tissue, which was finally worked out, is as follows:

Weigh accurately from 2 to 4 gm. of tissue in 50 cc. Pyrex Erlenmeyer flasks. The tissue must be analyzed wet, as drying in the oven at 100° or above introduces a loss of chlorides. It was found that this lost chloride could not be recovered as stated by Sunderman and Williams (2).

Add 5 cc. of 5 N potassium hydroxide and place the mixture in an oven set at 100–110° until solution occurs. This usually requires 15 to 30 minutes. As little potassium hydroxide solution as possible should be used, as it is desirable to keep the volume to a minimum at the time of titration. This permits the entire procedure to be carried out in the original flask. Also, the potassium hydroxide solution used should be as weak as possible and still give good solution. Otherwise, after neutralization with nitric acid, potassium nitrate will crystallize out. This can be redissolved, if it does form, by adding a small volume of distilled water. At this stage three or four control tubes should be prepared, carrying the same amount of potassium hydroxide as was used on the tissue. These serve as controls over the reagents.

Allow the solutions to cool. Determine the amount of concentrated nitric acid necessary to neutralize the potassium hydroxide by titrating one of the blank control tubes, methyl orange being used as indicator. Add a slight excess over the amount required for neutralization to each of the remaining flasks. Be careful that they do not foam over, either when the acid is added or during the subsequent redigestion. A drop or two of caprylic alcohol is sometimes advisable to prevent this. The solutions should be absolutely cold when the acid is added and the temperature of the flasks raised in the oven slowly to 100–110°. Redigest the solutions in the oven until they are all a clear yellow color. This usually requires from a few minutes to several hours. This oxidation in acid solution must be complete, or the solutions will regain their color on subsequent neutralization.

Allow the solutions to cool. Adjust their reaction to pH 7.0 to 7.5 with a drop or two of universal indicator. It is advisable to do this on the controls first to secure an approximate idea of the amount of base necessary and also to acquaint one with the color changes of the indicator. Too concentrated acid or base will

ruin the indicator so caution must be used. This may be avoided by first titrating a blank control tube to determine approximately the volume of potassium hydroxide required for neutralization and then adding this quantity to each tube in the series before adding the indicator. I usually roughly balance the solutions with 2 N nitric acid and potassium hydroxide and finish with 0.02 N strengths, using ordinary dropping pipettes. This is the crucial step in the procedure. The solutions must be accurately adjusted to pH 7.0 to 7.5 or the test will fail. The color should be a greenish yellow, but, if anything, nearer a light green than a yellow.

Add a drop or two of 0.1 per cent dichlorofluorescein to each flask and titrate with the standard silver nitrate solution. At the end-point the solution apparently loses its fluorescence and acquires a salmon-pink color. The titration should be carried out by reflected and not transmitted light, so that this fluorescence may be observed. Too much indicator should not be used, as this tends to obscure the end-point. If the end-point should be in doubt, the precipitate should be allowed to settle for a few seconds. At the end-point the precipitated silver chloride assumes a pinkish color owing to adsorption of the dye. The amount of silver nitrate used should be read to at least 0.01 cc. For this I find a 2 cc. burette graduated to 0.001 cc. very convenient, although an ordinary 10 cc. burette graduated to 0.05 cc. can be used. If the 2 cc. burette is used, it should be one equipped with a reservoir. In all events, all measuring instruments should be of the greatest accuracy.

Calculation

$$\frac{\text{Cc. standard silver nitrate used} \times 10}{\text{Weight of sample}} = \text{gm. NaCl per kilo}$$

The accuracy of the method was tested chiefly against the Sunderman-Williams (3) procedure and gravimetrically (Table I). It was tried on both normal tissue from various organs of the cat and on tissue to which known amounts of sodium chloride were added.

Analyses were made on liver, muscle, lung, and skin to which sodium chloride was added. Various amounts of standard 1 per cent sodium chloride were added to the samples. The theoretical amount of silver nitrate required for this added chloride was sub-

TABLE I
Chlorides in Tissue

The values are given in gm. of NaCl per kilo with the exception of those for whole blood, which are given in gm. per liter.

	Sunderman-Williams method (3)			Gravimetric method			Adsorption method		
	Cat 1	Cat 2	Cat 3	Cat 1	Cat 2	Cat 3	Cat 1	Cat 2	Cat 3
Liver.....	2.75	2.81	2.79	2.78	2.85	2.81	2.77	2.83	2.77
Kidney.....	4.25	4.04	4.27	4.30	4.25	4.31	4.27	4.19	4.33
Muscle.....	1.49	1.46	1.44	1.51	1.50	1.61	1.50	1.50	1.60
Lung.....	4.28	4.09	4.26	4.32	4.28	4.25	4.31	4.20	4.26
Heart.....	4.72	4.59	4.62	4.79	4.65	4.75	4.79	4.64	4.80
Pancreas.....	3.31	3.18	3.28	3.35	3.22	3.40	3.32	3.20	3.36
Skin.....	3.76	3.82	3.90	3.80	3.87	3.88	3.77	3.86	3.86
Spleen.....	3.18	3.33	3.31	3.25	3.40	3.35	3.23	3.39	3.31
Salivary glands.....	2.89	2.94	2.93	2.93	2.97	2.93	2.91	2.95	2.91
Brain (cerebrum).....	2.36	2.48	2.51	2.40	2.53	2.55	2.39	2.48	2.54
Whole blood.....	4.61	4.90	5.00	4.69	4.99	5.00	4.67	4.96	5.00

TABLE II
Recovery of Chlorides from Tissue

The values are given in gm. of NaCl per kilo.

	Sunderman- Williams method (3)	Gravimetric method	Adsorption method
Liver.....	2.79	2.85	2.83
“ + 1 cc. 1% NaCl.....	2.81	2.85	2.84
“ + 2 “ 1% “.....	2.84	2.85	2.85
Muscle.....	1.50	1.58	1.56
“ + 1 cc. 1% NaCl.....	1.59	1.57	1.58
“ + 2 “ 1% “.....	1.71	1.58	1.60
Lung.....	4.22	4.39	4.36
“ + 1 cc. 1% NaCl.....	4.40	4.40	4.40
“ + 2 “ 1% “.....	4.57	4.40	4.49
Skin.....	3.87	3.96	3.90
“ + 1 cc. 1% NaCl.....	3.98	3.97	3.96
“ + 2 “ 1% “.....	4.03	3.99	4.00

tracted from the total amount used and the result used in calculating the amount of chloride in the tissue. The results are tabulated in Table II.

After the method for the determination of chloride in tissue was perfected, it was quite simple to adapt it to blood and urine (Tables III and IV).

Blood chloride may be determined by the above method, a digest of 2 cc. of whole blood being used. It is usually simpler to use 10 cc. of the usual protein-free filtrate, however. If the filtrate is used, a blank must be run on the protein-precipitating reagents

TABLE III
Chlorides in Blood

The values are given in mg. per 100 cc.

Whitehorn method (4)			Gravimetric method			Adsorption method		
Cat	Dog	Human	Cat	Dog	Human	Cat	Dog	Human
450	500	443	469	512	457	466	509	452
490	493	469	503	500	491	500	500	486
472	502	490	482	510	500	480	500	501
505	488	483	516	495	494	510	490	490
485	475	461	500	475	465	485	482	466

TABLE IV
Chlorides in Urine

The values are given in gm. per 100 cc. These are results taken at random from a large number of determinations.

Volhard-Arnold method (5)			Gravimetric method			Adsorption method		
Cat	Dog	Human	Cat	Dog	Human	Cat	Dog	Human
1.21	1.26	1.00	1.27	1.30	1.06	1.25	1.29	1.05
1.02	1.19	1.29	1.15	1.25	1.31	1.13	1.26	1.35
1.18	1.09	1.23	1.25	1.11	1.30	1.26	1.09	1.30
1.07	1.03	1.22	1.20	1.14	1.24	1.17	1.11	1.25
1.13	1.18	1.18	1.18	1.26	1.24	1.20	1.23	1.20

to determine their chloride content. The filtrate or digest is adjusted to pH 7.0 to 7.5 and titrated exactly as in the procedure for tissue digests.

Calculation

Cc. standard silver nitrate \times 500 = mg. NaCl per 100 cc. blood

Urine chloride is determined by the following method. Exactly 1 cc. of urine is diluted with about 5 cc. of distilled water. The

solution is adjusted to pH 7.0 to 7.5 in the usual manner. It is then titrated with standard silver nitrate and dichlorofluorescein exactly as with tissue digests. The volume of standard silver nitrate used, in cc., is equal to the number of gm. of sodium chloride in 100 cc. of that urine (*i.e.*, if 1.12 cc. of the standard silver nitrate was used, then 100 cc. of the urine contains 1.12 gm. of sodium chloride).

SUMMARY

The method proposed for the determination of chlorides in biological materials is shorter and simpler to use than the older silver nitrate-thiocyanate procedures. It is extremely accurate if ordinary care and accurate measuring instruments are used. It reduces the possibilities of error by eliminating filtration and changes from one container to another. It also reduces the possibility of error from faulty standard solutions inasmuch as only one standard solution is used.

I should like to acknowledge my appreciation to Mr. Stephen Krop of the Department of Pharmacology for a suggestion concerning Fajans' technique and to Mr. C. S. Moore for his valuable assistance in preparing the tables accompanying this paper.

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ON THEVETIN

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The constituents of be-still nuts, the fruit of the plant *Thevetia neriifolia*, have recently been critically examined by Chen and Chen.¹ They succeeded in isolating three crystalline substances: viz., thevetin, to which the formula $C_{29}H_{46}O_{13}$ was given; ahouain, of the formula $C_{10}H_{19}O_{10}$; and kokilphin, $C_{33}H_{61}O_{30}$. Of these, thevetin was shown to possess the pharmacodynamic properties typical of the digitalis group of cardiac poisons.² This relationship was further strengthened by the observation that thevetin gave the positive nitroprusside (Legal) reaction characteristic of the members of the digitalis-strophanthus group.

For the historical background, reference should be made to Chen and Chen's paper.¹ Since its appearance, Ghatak³ has reiterated his opinion that thevetin can best be represented by the formula $C_{20}H_{30}O_6$, and that on hydrolysis, glucose and a genin, $C_{14}H_{20}O$, result.

Through the kindness of Dr. K. K. Chen of The Lilly Research Laboratories, Indianapolis, who has kindly supplied generous amounts of crystalline thevetin, it has become possible to characterize the glycoside more fully and to demonstrate more conclusively its intimate relationship to the other members of the digitalis group.

The sugar component of thevetin has been shown, after hydrolytic cleavage, to be *glucose* by its isolation as *glucosazone* and by the failure to obtain mannose phenylhydrazone. The aglycone

¹ Chen, K. K., and Chen, A. L., *J. Biol. Chem.*, **105**, 231 (1934).

² Chen, K. K., and Chen, A. L., *J. Pharmacol. and Exp. Therap.*, **51**, 23 (1934).

³ Ghatak, N., *Proc. Acad. Sc. Agra and Oudh, India*, **4**, 173 (1934).

portion of the molecule would then possess the formula $C_{23}H_{36}O_8$ if the formulation for thevetin adopted by Chen and Chen be accepted. However, if, as is indicated by the positive nitroprusside reaction, the characteristic $\Delta^{\beta,\gamma}$ -unsaturated lactone side chain is present, the formula for the aglycone, thevetogenin, should be revised to $C_{23}H_{34}O_8$ and that for thevetin would become $C_{29}H_{44}O_{13}$. Thevetogenin would thus be isomeric with ouabagenin, the hypothetical aglycone of ouabain.⁴ This revision is supported by the fact that thevetin on catalytic hydrogenation absorbs 1 mole of hydrogen, although it has not been possible to obtain dihydrothevetin in crystalline form, and by the formation of *isothevetin* when thevetin is subjected to the isomerizing action of alkali. These reactions again are characteristic of the unsaturated lactone side chain of the cardiac aglycones.

Attention was next directed to an examination of the aglycone. Because of the presence of the normal hexose, glucose, rather than a 2-desoxy sugar, hydrolysis of thevetin itself required sufficiently strenuous treatment to destroy the aglycone. However, after isomerization to isothevetin, it has been possible to obtain crystalline derivatives of thevetogenin. Manipulation was rendered exceedingly difficult by the tendency of the derivatives to crystallize in a highly hydrated state and frequently only in the presence of electrolytes which prevented their isolation in a state of purity.

When acetolysis was attempted with isothevetin by the procedure which had proved successful with isoouabain,⁵ only non-crystalline resins resulted. However, when isothevetin was carefully warmed with 2 per cent HCl, a less soluble crystalline substance separated from the solution. The analytical data indicated a *monohydrate of isothevetogenin*, but manipulation was made difficult by its unfortunate physical properties.

Better success attended the hydrolysis of the glycoside after oxidation with hypobromite. When isothevetin was oxidized in this manner after saponification of the lactone group, an acid, *isothevetinic acid*, was formed. This readily crystallized in the presence of electrolytes, such as NaCl, but when attempts were made to wash it free from salts, it assumed gelatinous properties. However, gentle acid hydrolysis of the crude isothevetinic acid

⁴ Jacobs, W. A., and Bigelow, N. M., *J. Biol. Chem.*, **96**, 647 (1932).

⁵ Jacobs, W. A., and Bigelow, N. M., *J. Biol. Chem.*, **101**, 15 (1933).

resulted in the formation of crystalline *isothevetogenic acid* which was brought to a state of analytical purity, although with difficulty. Attempts to prepare a methyl- or *p*-bromophenacyl ester of this acid resulted in gelatinous products. Benzoylation of the amorphous methyl ester led to an amorphous benzoate and acetylation gave an acetate which crystallized when strongly chilled, but melted at room temperature during manipulation. Likewise, attempts to characterize the hydroxyl groups by oxidation to carbonyl resulted in non-crystalline products.

These results confirm the placing of thevetin in the digitalis-strophanthus group of cardiac aglycones. The sugar has been shown to be glucose and the aglycone, while not isolated as such, has been shown to be isomeric with ouabagenin and exhibits the typical reactions of the other members of the group. While it is not possible, with the information at hand, to place the hydroxyl groups of thevetogenin definitely, analogy with the other members of the group warrants the assumption of the presence of a secondary hydroxyl group on carbon atom (3) and of a tertiary hydroxyl group on carbon atom (14).

EXPERIMENTAL

Thevetin—The glucoside as received was recrystallized two times from 85 per cent isopropyl alcohol. It softened at 193–194° and slowly went to bubbles up to 210°. For analysis it was dried at 80° and 0.1 mm.

$C_{25}H_{44}O_{13}$	Calculated.	C 57.96,	H 7.40
	Found.	" 57.90, 57.69,	" 7.59, 7.46

Isothevetin—1 gm. of thevetin was dissolved in 10 cc. of absolute methyl alcohol and the solution chilled in ice. 10 cc. of a 20 per cent solution of potassium hydroxide in absolute methyl alcohol were added and the mixture was allowed to warm up to room temperature. After 30 minutes, the Legal test was negative. 100 cc. of water were added and the solution was made just acid to Congo red with HCl. After standing 4 hours at room temperature to complete lactonization, the solution was neutralized to Congo red with sodium acetate and concentrated *in vacuo* to copious crystallization. The crystalline material was centrifuged and recrystallized twice from water. Isothevetin as thus obtained

forms micaceous leaflets which were highly hydrated and extremely difficult to manipulate. For analysis it was recrystallized from isopropyl alcohol, from which it separates as microleaflets. After drying in a desiccator over P_2O_5 , the substance still retained 0.5 mole of solvent. It shrinks at 180° and goes to a mass of fine bubbles at $200\text{--}210^\circ$.

$$[\alpha]_D^{24} = -60^\circ \quad (c = 0.866 \text{ in pyridine})$$

For analysis the substance was dried at 100° and 0.2 mm.

$C_{29}H_{44}O_{13}$.	Calculated.	C 57.96,	H 7.40
	Found.	" 57.80, 58.05,	" 7.63, 7.65

12.130 mg. of substance were refluxed with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and titrated back against phenolphthalein with 0.1 N HCl. 0.214 cc. of 0.1 N NaOH was consumed. Calculated for 1 equivalent, 0.202 cc.

Isothevetogenin—130 mg. of isothevetin were heated in 10 cc. of 2 per cent HCl on the steam bath for 5 minutes. During this time the isothevetin dissolved and was replaced by lens-shaped crystals of the isogenin. These were filtered from the cooled solution and recrystallized from dilute methyl alcohol. On drying in a desiccator, the crystals shrank and effervesced to a varnish. In this form it sintered at about 170° and decomposed at 220° , although these temperatures varied greatly with the rate of heating. The Molisch test was negative. For analysis it was further dried at 100° and 0.2 mm. over H_2SO_4 . The figures obtained indicated that it still retained 1 mole of H_2O .

$C_{23}H_{34}O_8 \cdot H_2O$.	Calculated.	C 60.49,	H 7.95
	Found.	" 60.33, 60.96,	" 7.56, 7.77

Isothevetinic Acid—1 gm. of isothevetin was dissolved in 25 cc. of water containing 2.5 cc. of 10 per cent NaOH solution. 50 cc. of a solution of NaOBr (prepared from 50 cc. of N NaOH and 3.3 gm. of Br) were added. After standing 1 hour at room temperature, the alkaline solution was extracted with ether in order to remove the CBr_4 formed by oxidation of the isopropyl alcohol of crystallization of the isothevetin. The solution was then made acid to litmus with acetic acid and evaporated to about 15 cc. in a desiccator. It was then acidified to Congo red

with HCl. After 2 hours the fine prisms which separated were centrifuged off. The substance is very highly hydrated and crystallized only from diluted solvents in the presence of inorganic electrolytes. When attempts were made to wash it free of salts, the substance became gelatinous as the salts were removed. In the crystalline state it adsorbed too large an amount of salts to permit accurate analytical results. However, after removal of the sugar the resulting acid was purified, although with difficulty.

Isothevetogenic Acid—The alkaline solution from the oxidation of 1 gm. of isothetvetin, as described above, was made faintly but distinctly acid to Congo red with HCl and then heated in a beaker of actively boiling water for 10 minutes. During this time a turbidity formed and fine needles separated. The solution was cooled as rapidly as possible and the crystals were centrifuged. The substance appeared to crystallize only from aqueous solvents, best from dilute acetone. Addition of a few drops of acetic acid was found to facilitate crystallization. The crystals were apparently highly hydrated and very difficult to filter. Therefore, they were centrifuged and dried as such in a desiccator, during which large amounts of water were lost and the substance dried down to a resin. In this state it froths up at 120–130° and slowly decomposes.

$$[\alpha]_D^{25} = -47^\circ \quad (c = 1.075 \text{ in pyridine})$$

For analysis it was further dried at 100° and 20 mm.

$C_{23}H_{34}O_9$.	Calculated.	C 60.76,	H 7.54
	Found.	" 60.29, 60.48,	" 7.74, 7.86

The original acid mother liquor from the above was again heated at 100° for 10 minutes and an additional amount of isothetvetogenic acid obtained. Further hydrolysis resulted in the formation of obscure dehydration products.

Isolation of Glucosazone from Thevetin—0.5 gm. of thevetin was heated on the steam bath with 30 cc. of 3 per cent H_2SO_4 for 5 hours. The mixture was chilled and filtered from resinous hydrolysis products. Excess $BaCO_3$ was then added and the solution was again filtered. 15 cc. of 50 per cent $NaHSO_3$ solution, 15 cc. of a saturated solution of sodium acetate in 50 per cent acetic acid, and 1.5 cc. of phenylhydrazine were added to the

filtrate, and the mixture was heated 1 hour on the steam bath. After cooling, the osazone was collected and recrystallized from methyl alcohol. It decomposed at 207° and showed no depression when mixed with glucosazone.

A 1 per cent solution of the osazone in 2:3 pyridine-alcohol solution at first showed $\alpha_D^{28} = -0.55^{\circ}$ in a 1 dm. tube. After 24 hours, $\alpha_D^{28} = -0.35^{\circ}$. This is in accord with the observed mutarotation of glucosazone.⁶

$C_{18}H_{22}O_4N_4$.	Calculated.	C 60.30,	H 6.19
	Found.	" 60.22, 60.31,	" 6.31, 6.41

In another experiment, the sugar solution from the hydrolysis of 100 mg. of thevetin was similarly treated with phenylhydrazine and allowed to stand at room temperature. No mannose phenylhydrazone was formed.

⁶ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **20**, 429 (1915).

OPTICAL ROTATIONS AND ROTATORY DISPERSIONS IN HOMOLOGOUS SERIES OF ALIPHATIC NITRILES

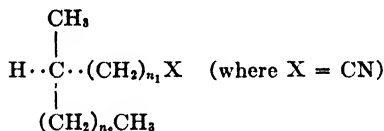
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The present communication deals with the optical rotation of nitriles of the general formula



The interest attached to these substances lies in the character of the chromophoric group of the radicle $-\text{C} \equiv \text{N}$. Nitriles may be regarded as the anhydrides of ammonocarboxylic acids and yet the nearest absorption band of the $-\text{C} \equiv \text{N}$ group (apparently continuous) is located in the Schumann region at $\approx \lambda$ 1600. Discontinuous bands start at wave-lengths shorter than λ 1450.¹ The point of interest, then, is the course of events resulting from the progressive increase in the value of n_1 , on one hand, and of n_2 , on the other.

In Table III are given the maximum rotations and in Table IV the rotatory dispersions of the configurationally related nitriles. Their configurational relationship was established on the basis of their preparation from acids or from halides of known configuration. The rotations of the acid chlorides and of the corresponding amides are given in Tables I and II.

In the series homologous with respect to n_2 , the course of events changes with the variation in the value of n_1 . In the series having

¹ Herzberg, G., and Scheibe, G., *Z. physik. Chem., Abt. B*, **7**, 390 (1930).

TABLE I
Configurally Related Aliphatic Acid Chlorides. $[M]_D^{25}$ Maximum (Homogeneous)

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{COCl} \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COCl} \\ \\ \text{C}_2\text{H}_5 \\ +18^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{COCl} \\ \\ \text{C}_2\text{H}_5 \\ +19^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{COCl} \\ \\ \text{C}_2\text{H}_5 \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{COCl} \\ \\ \text{C}_3\text{H}_7 \text{ (n)} \\ +14 \text{ } 4^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COCl} \\ \\ \text{C}_3\text{H}_7 \text{ (n)} \\ +4 \text{ } 6^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{COCl} \\ \\ \text{C}_3\text{H}_7 \text{ (n)} \\ +6 \text{ } 8^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{COCl} \\ \\ \text{C}_3\text{H}_7 \text{ (n)} \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{COCl} \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \\ \text{Dextro} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COCl} \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \\ +3.5^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{COCl} \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{COCl} \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \\ +3.5^\circ \end{array}$

TABLE II
Configurally Related Acid Amides. $[M]_D^{25}$ (75 Per Cent Alcohol)

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CONH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +19^{\circ*} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CONH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +5.7^{\circ\dagger} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CONH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +19^{\circ} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CONH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +1.6^{\circ} \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CONH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ +24^{\circ\dagger} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CONH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ -6.8^{\circ\dagger} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CONH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ \sim +6.6^{\circ} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CONH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CONH}_2 \\ \\ \text{C}_4\text{H}_9 (n) \\ +18^{\circ\dagger} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CONH}_2 \\ \\ \text{C}_4\text{H}_9 (n) \\ -9.9^{\circ\dagger} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CONH}_2 \\ \\ \text{C}_4\text{H}_9 (n) \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CONH}_2 \\ \\ \text{C}_4\text{H}_9 (n) \\ +1.6^{\circ} \end{array}$

* Taverne, H. J., *Rec. trav. chim. Pays-Bas*, **19**, 107 (1900).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

‡ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 571 (1929).

TABLE III
Configurationally Related Aliphatic Nitriles. $[M]_D^{25}$ Maximum (Homogeneous)

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CN} \\ \\ \text{C}_2\text{H}_5 \\ +25^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CN} \\ \\ \text{C}_2\text{H}_5 \\ +8.7^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CN} \\ \\ \text{C}_2\text{H}_5 \\ +17^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CN} \\ \\ \text{C}_2\text{H}_5 \\ +17^\circ \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CN} \\ \\ \text{C}_2\text{H}_7 (n) \\ +49^{**} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CN} \\ \\ \text{C}_2\text{H}_7 (n) \\ +4.6^\circ \dagger \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CN} \\ \\ \text{C}_2\text{H}_7 (n) \\ +2.0^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CN} \\ \\ \text{C}_2\text{H}_7 (n) \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CN} \\ \\ \text{C}_4\text{H}_9 (n) \\ +51^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{CN} \\ \\ \text{C}_4\text{H}_9 (n) \\ +6.0^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{CN} \\ \\ \text{C}_4\text{H}_9 (n) \\ -1.6^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{CN} \\ \\ \text{C}_4\text{H}_9 (n) \\ +5.3^\circ \end{array}$
		$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{CN} \\ \\ \text{C}_4\text{H}_9 (n) \\ +2.0^\circ \end{array}$	

* Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 571 (1929).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

$n_1 = 0$, the rotations of the substances given in Table III increase progressively and remain of the same direction as that of the first member. In this respect the nitriles reflect fully the events of the parent carboxylic acids. They differ from the latter by the higher values of their molecular rotations.

In the series having $n_1 = 1$, there is observed one unexplainable irregularity. In the many homologous series analyzed to date, the rotations of successive members shifted progressively either to the right or to the left. In the homologous series of nitriles having $n_1 = 1$, the shift of the maximum molecular rotation is to the left between the first two members, whereas on passing from $n_2 = 2$ to $n_2 = 3$, it is to the right. This peculiarity cannot be attributed to experimental error for the reason that two series of experiments were performed independently with identical results, and, furthermore, on reduction of the nitriles to the corresponding amines, the latter form a perfectly normal homologous series. In the series of nitriles having $n_1 = 2$ and $n_1 = 3$, given in Table III, the rotations of the individual members increase progressively to the left, changing in sign in the higher members, in a manner similar to that of the series of aliphatic carboxylic acids. These events indicate that the rotations of the nitriles of those series are the resultant of two partial rotations of opposite sign.

In the series homologous with respect to n_1 , the change in rotation with the progressive increase in the value of n_1 is of particular interest for the reason that two events have been observed when

$X = -\text{COOH}$, or $-\text{C} \begin{array}{c} \text{O} \\ \parallel \\ \text{H} \end{array}$; first, a change in the direction of the

partial rotations of the functional group on passing from $n_1 = 0$ to $n_1 = 1$; second, a periodicity in the direction of the shift of rotation with the further increase in the value of n_1 . From the first and second columns of Table III it can be noted that from $n_1 = 0$ to $n_1 = 1$, there is a drop in the values of the rotation without change of sign. A periodicity in the shift of rotation in the visible region of the spectrum is observed in the substances given in the first row (the lowest homologues). In the substances given in the third and fourth rows, the periodicity begins with the

TABLE IV
Rotatory Dispersions of Configurationally Related Nitriles in Heptane Solution

λ	Dextro-2-methylhexanenitrile. Constants: $d_4^{25} = 0.7962$; $n_D^{25} = 1.4062$; concentration, 0.987 M; $l = 40$ cm. in visible and 10 cm. in ultraviolet region		Dextro-3-methylheptanenitrile. Constants: $d_4^{25} = 0.8104$; $n_D^{25} = 1.4181$; concentration, 0.7277 M; $l = 100$ cm.		Levo-4-methyloctanenitrile. Constants: $d_4^{25} = 0.8164$; $n_D^{25} = 1.4245$; concentration, 0.8459 M; $l = 100$ cm.		Dextro-5-methylnonanitrile. Constants: $d_4^{25} = 0.8187$; $n_D^{25} = 1.4290$; concentration, 0.7131 M; $l = 100$ cm.		Dextro-6-methyldecanenitrile. Constants: $d_4^{25} = 0.8207$; $n_D^{25} = 1.4320$; concentration, 0.7609 M; $l = 100$ cm.	
	α_D^{25}	$[M]_{\max}^{25}$	α_D^{25}	$[M]_{\max}^{25}$	α_D^{25}	$[M]_{\max}^{25}$	α_D^{25}	$[M]_{\max}^{25}$	α_D^{25}	$[M]_{\max}^{25}$
5875.6	4.072	52.53	3.099	6.833	-2.059	-2.619	1.951	5.431	0.769	1.976
5780.1	4.225	54.50	3.222	7.104	-2.126	-2.704	2.019	5.620	0.801	2.058
5460.7	4.787	61.75	3.637	8.020	-2.371	-3.016	2.281	6.349	0.901	2.327
4358.3	7.96	102.7	5.94	13.10	-3.685	-4.69	3.80	10.58	1.54	3.96
4046.6	9.50	122.5	6.99	15.41	-4.25	-5.40	4.54	12.64	1.85	4.75
3550	3.30	170	1.30, λ 2400 ($l = 5$ cm.)	57.5	-0.65 from λ 3200 to λ 2700 ($l = 10$ cm.)	0.65, λ 3510 0.90, λ 3125 ($l = 10$ cm.)	18.1	25.0	0.90, λ 2450 ($l = 10$ cm.)	23.1
3360	3.80	196								
3215	4.30	222								
3090	4.80	247.5								
2990	5.30	273.5								
2885	5.80	299								
2750	6.80	351								
2585	8.30	428								
2350	1.15 ($l = 1$ cm.)	593								

member having $n_1 = 1$. However, if the partial rotations of the CN groups are compared, then it is found that a change of direction occurs on passing from $n_1 = 0$ to $n_1 = 1$.

A complete analysis of the total rotations of nitriles into their different partial components is impossible for the reason that the absorption bands of the chromophoric group are too distant from the smallest wave-lengths accessible to rotatory measurements. In fact, the rotatory dispersion curves of all the nitriles (with the exception of 4-methyloctanenitrile) can be expressed from the visible region to about λ 2400 by a single Drude term within the limits of error. It should be stated, however, that the relative precision attained is low on account of the small values of the molecular rotations (see Table IV and related dispersion formulæ).

In the case of the nitriles having $n_1 = 0$, the dispersion constant corresponds to the position of the first absorption band, and the molecular rotations are high. These facts indicate that the main contribution is furnished by the —CN group. The slight deviation from linearity of the function $1/[M] = f(\lambda^2)$ seems to indicate that the second term is of the sign of the first term.

The rotations of the primary nitrile with $n_1 = 1$ are low and the constant of dispersion of the one Drude term formula is λ 1380; that is, 260 Å. lower than that of the secondary nitrile. This fact seems to show that the dispersion of the compound is anomalous; *i.e.*, the sign of rotation of the first active band is opposite to the rotation observed inasmuch as other substances with $n_1 = 1$ having a normal dispersion curve are as a rule very dispersive (their dispersion constants of a one Drude term formula have generally a higher value than that corresponding to the first active absorption band).

The primary nitrile with $n_1 = 2$ has a low rotation too and the course of its dispersion is typically anomalous. The constant of dispersion is negative and there is observed a broad flat minimum in the function $1/[M] = f(\lambda^2)$ from λ 3200 to λ 2700. The lower homologues of this series rotate in opposite direction and although dispersion measurements have not been made on them, it is almost certain that their dispersion is normal. In fact, their dispersion power should be the highest of all the nitriles. The nitriles with $n_1 = 3$ or 4 exhibit a normal dispersion and their constants of

dispersion are greater than the position of the first absorption band in agreement with the view that the two main contributions are of opposite sign.

The rule previously enunciated, that for two homologues (with respect to n_1 or n_2) possessing the same type of dispersion, the one with the greater dispersive power has the smaller maximum molecular rotation, has been verified in the case of the nitriles.

The conclusion, then, is that the effect of the progressive increase of the value of n_1 in the series of aliphatic nitriles is very similar to that in the series of aliphatic carboxylic acids and in the series of aliphatic aldehydes.

Effect of the Solvent—From Table V it can be seen that the total rotation of each nitrile differs, depending upon the medium, as a

TABLE V

$$\begin{array}{c} \text{CH}_3 \\ | \\ \text{H} \cdot \text{C} \cdot (\text{CH}_2)_n \text{CN} \\ | \\ \text{C}_6\text{H}_5 \end{array}$$

 Rotations of Nitriles of the Type $\text{H} \cdot \text{C} \cdot (\text{CH}_2)_n \text{CN}$ in Homogeneous State
 and in Heptane

n_1	0	1	2	3	4
Maximum $[\text{M}]_D^{25}$ (homogeneous).....	50.5	5.94	-1.56	5.31	1.95
“ “ (heptane).....	52.3	6.82	-2.62	5.43	1.97

rule being higher in heptane solution. It is worthy of note that the absolute differences are of the same order of magnitude in all members.

Equations Expressing the Rotatory Dispersions of Nitriles

Dextro-2-methylhexanenitrile.	$[\text{M}]_{\text{max}}^{25}$	$\frac{16.742}{\lambda^2 - 0.0269}$
Dextro-3-methylheptanenitrile.	“	$\frac{2.2382}{\lambda^2 - 0.0191}$
Levo-4-methyloctanenitrile.	“	$\frac{-0.9214}{\lambda^2 - 0.0066}$
Dextro-5-methylnonanenitrile.	“	$\frac{1.7241}{\lambda^2 - 0.0273}$
Dextro-6-methyldecanenitrile.	“	$\frac{0.6166}{\lambda^2 - 0.034}$

EXPERIMENTAL

Levo-2-Methylbutanenitrile—75 gm. of 2-methylbutanoic acid, $[\alpha]_D^{25} = -2.1^\circ$ (homogeneous), were converted to the nitrile through the acid chloride and amide. Yield 20 gm. B.p. 127° at 760 mm.

$$[\alpha]_D^{25} = \frac{-2.80^\circ}{1 \times 0.806} = -3.47^\circ; [\text{M}]_D^{25} = -2.88^\circ$$

$$\text{Maximum } [\text{M}]_D^{25} = -24.7^\circ \text{ (homogeneous)}$$

3.688 mg. substance: 9.900 mg. CO_2 and 3.530 mg. H_2O

4.794 " " : 0.647 cc. N_2 at 23° and 768 mm.

$\text{C}_5\text{H}_9\text{N}$. Calculated. C 72.2, H 10.9, N 16.9

83.1 Found. " 73.2, " 10.7, " 15.7

*Dextro-3-Methylpentanenitrile*²—25 gm. of 1-bromo-2-methylbutane,

$$[\alpha]_D^{25} = \frac{+1.40^\circ}{1 \times 1.22} = +1.15^\circ \text{ (homogeneous)}$$

(from Kahlbaum's amyl alcohol $\alpha_D^{25} = -1.75^\circ$ (1 dm.) (homogeneous)), were added to a solution of 12 gm. of potassium cyanide (1.1 moles) in 20 cc. of water and 150 cc. of ethanol. This solution was refluxed for 48 hours. It was then poured into water, concentrated calcium chloride solution was added, and the solution was extracted with ether. The extract was washed with concentrated calcium chloride solution, dried, and the substance was distilled. B.p. 154° , p = atmospheric. Yield 9.5 gm.

$$[\alpha]_D^{25} = \frac{+1.60^\circ}{1 \times 0.811} = +1.97^\circ; [\text{M}]_D^{25} = +1.91^\circ$$

$$\text{Maximum } [\text{M}]_D^{25} = +8.67^\circ \text{ (homogeneous)}$$

4.700 mg. substance: 12.760 mg. CO_2 and 4.730 mg. H_2O

$\text{C}_6\text{H}_{11}\text{N}$. Calculated. C 74.2, H 11.4

97.1 Found. " 74.0, " 11.3

Dextro-4-Methylhexanoic Chloride—50 gm. of 4-methylhexanoic acid, $[\text{M}]_D^{25} = +1.6^\circ$, were treated with 100 gm. of thionyl chloride. This solution was refluxed for 2 hours. The excess thionyl chloride was distilled off. The product was then distilled. B.p. 80° at 50 mm. Yield 45 gm.

² Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

$$[\alpha]_D^{25} = \frac{+1.46^\circ}{1 \times 0.974} = +1.50^\circ; [M]_D^{25} = +2.23^\circ$$

Maximum $[M]_D^{25} = +19.0^\circ$ (homogeneous)

3.011 mg. substance: 6.182 mg. CO_2 and 2.120 mg. H_2O

4.700 " " : 4.610 " AgCl

$\text{C}_7\text{H}_{13}\text{OCl}$. Calculated. C 56.5, H 8.8, Cl 23.9

148.6 Found. " 56.0, " 8.0, " 24.2

Dextro-4-Methylhexaneamide—45 gm. of 4-methylhexanoic chloride, $[M]_D^{25} = +2.23^\circ$, were dropped slowly into aqueous ammonia at -10° . The amide was worked up as usual, and after recrystallization had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.19^\circ \times 100}{1 \times 20 \times 0.570} = +1.67^\circ; [M]_D^{25} = +2.17^\circ$$

Maximum $[M]_D^{25} = +19.1^\circ$ (75% alcohol)

Dextro-4-Methylhexanenitrile—40 gm. of 4-methylhexaneamide, $[M]_D^{25} = +2.23^\circ$ (75 per cent alcohol), were heated with 60 gm. of phosphorus pentoxide. The nitrile was then distilled at reduced pressure. Yield 20 gm. after purification. B.p. 105° at 100 mm.

$$[\alpha]_D^{25} = \frac{+1.45^\circ}{1 \times 0.817} = +1.77^\circ; [M]_D^{25} = +1.97^\circ$$

Maximum $[M]_D^{25} = +16.7^\circ$ (homogeneous)

A duplicate preparation gave the same readings.

4.790 mg. substance: 0.534 cc. N_2 at 19° and 741 mm.

$\text{C}_7\text{H}_{13}\text{N}$ (111.1). Calculated, N 12.60; found, N 12.70

Dextro-5-Methylheptanenitrile—30 gm. of 1-bromo-4-methylhexane,

$$[\alpha]_D^{25} = \frac{+4.50^\circ}{1 \times 1.129} = +3.99^\circ \text{ (homogeneous)}$$

were added to a solution of 12 gm. of potassium cyanide in 30 cc. of water and 120 gm. of absolute ethanol. The solution was refluxed for 38 hours. The nitrile was isolated as described. B.p. $115-133^\circ$, $p = 98$ to 100 mm. Yield 17 gm. $d_4^{25} = 0.8039$ (*in vacuo*). $n_D^{25} = 1.4175$.

$$[\alpha]_D^{25} = \frac{+3.63^\circ}{1 \times 0.804} = +4.51^\circ; [M]_D^{25} = +5.64^\circ$$

$$\text{Maximum } [M]_D^{25} = +17.3^\circ \text{ (homogeneous)}$$

5.002 mg. substance: 14.125 mg. CO₂ and 5.340 mg. H₂O

C₈H₁₅N. Calculated. C 76.7, H 12.1

125.1 Found. " 76.9, " 11.9

Levo-4-Methylheptanoic Chloride—30 gm. of 4-methylheptanoic acid, $[M]_D^{25} = -1.72^\circ$ (homogeneous), were treated with excess of thionyl chloride as usual. The substance was then fractionated. B. p. 82° at 30 mm. Yield 30 gm. $d_4^{25} = 0.953$.

$$[\alpha]_D^{25} = \frac{-0.99^\circ}{1 \times 0.953} = -1.04^\circ; [M]_D^{25} = -1.69^\circ$$

$$\text{Maximum } [M]_D^{25} = -6.78^\circ \text{ (homogeneous)}$$

5.395 mg. substance: 4.600 mg. silver chloride

C₈H₁₅OCl (162.6). Calculated, Cl 21.2; found, Cl 21.09

Levo-4-Methylheptanenitrile—30 gm. of 4-methylheptanoic chloride, $[M]_D^{25} = -1.69^\circ$ (homogeneous), were converted into the nitrile through the amide. The product was distilled under reduced pressure. Yield 15 gm. B. p. 125° at 100 mm. $d_4^{25} = 0.818$.

$$[\alpha]_D^{25} = \frac{-0.33^\circ}{1 \times 0.818} = -0.40^\circ; [M]_D^{25} = -0.50^\circ$$

$$\text{Maximum } [M]_D^{25} = -2.01^\circ \text{ (homogeneous)}$$

4.275 mg. substance: 0.496 cc. N₂ at 21° and 739 mm.

C₈H₁₅N (125.1). Calculated, N 11.2; found, N 11.2

*Dextro-2-Methylhexanenitrile*³—This compound was prepared from 2-methylhexanoic acid,

$$[\alpha]_D^{25} = \frac{+3.40^\circ}{1 \times 0.909} = +3.74^\circ \text{ (homogeneous)}$$

³ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 571 (1929).

by the usual set of reactions. B.p. 75–77.5°, $p = 20$ mm. $d_4^{25} = 0.7962$ (*in vacuo*). $n_D^{25} = 1.4062$.

$$[\alpha]_D^{25} = \frac{+7.20^\circ}{1 \times 0.796} = +9.05^\circ; [M]_D^{25} = +10.1^\circ$$

$$\text{Maximum } [M]_D^{25} = +50.5^\circ \text{ (homogeneous)}$$

$$[\alpha]_{5875.6}^{25} = \frac{+4.071^\circ \times 100}{4 \times 10.75} = +9.47^\circ; [M]_{5875.6}^{15} = +10.5^\circ$$

$$\text{Maximum } [M]_{5875.6}^{25} = +52.5^\circ \text{ (heptane)}$$

4.530 mg. substance: 12.585 mg. CO₂ and 4.790 mg. H₂O

C₇H₁₃N. Calculated. C 75.59, H 11.81

111.1 Found. " 75.76, " 11.83

*Dextro-3-Methylheptanenitrile*²—3-Methylheptanoic acid,

$$[\alpha]_D^{25} = \frac{-2.40^\circ}{1 \times 0.909} = -2.64^\circ \text{ (homogeneous)}$$

was converted into the chloride, then into the amide, and finally into the nitrile as usual. B.p. 48–52°, $p = 0.5$ mm. $d_4^{25} = 0.8104$ (*in vacuo*). $n_D^{25} = 1.4181$.

$$[\alpha]_D^{25} = \frac{+2.40^\circ}{1 \times 0.810} = +2.96^\circ; [M]_D^{25} = +3.71^\circ$$

$$\text{Maximum } [M]_D^{25} = +5.97^\circ \text{ (homogeneous)}$$

$$[\alpha]_{5875.6}^{25} = \frac{+3.095^\circ \times 100}{10 \times 9.096} = +3.40^\circ; [M]_{5875.6}^{25} = +4.26^\circ$$

$$\text{Maximum } [M]_{5875.6}^{25} = +6.86^\circ \text{ (heptane)}$$

Dextro-4-Methyloctanenitrile—50 gm. of 1-bromo-3-methylheptane,

$$[\alpha]_D^{25} = \frac{+9.00^\circ}{1 \times 1.106} = +8.14^\circ \text{ (homogeneous)}$$

were added to a solution of 19 gm. of KCN in 32 cc. of water and 240 cc. of ethanol. This solution was refluxed for 2 days and the nitrile was isolated as usual. B.p. 119–120°, $p = 30$ mm. Yield 30 gm. $d_4^{25} = 0.8164$ (*in vacuo*). $n_D^{25} = 1.4245$.

$$1 \times 0.816$$

$$\text{Maximum } [M]_D^{25} = -1.56^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{-2.059^\circ \times 100}{10 \times 11.78} = -1.75^\circ; [M]_{6975.6}^{25} = -2.44^\circ$$

$$\text{Maximum } [M]_{6975.6}^{25} = -2.63^\circ \text{ (heptane)}$$

5.340 mg. substance; 0.478 cc. N_2 at 22° and 761 mm.

$C_9H_{17}N$ (139.2). Calculated, N 10.1; found, N 10.3

Levo-5-Methylnonanoic Chloride—50 gm. of 5-methylnonanoic acid, $[M]_D^{25} = -0.99^\circ$ (homogeneous), were treated with 100 gm. of thionyl chloride in the usual way. The product was then distilled. B.p. 111° at 14 mm. $d_4^{25} = 0.970$.

$$[\alpha]_D^{25} = \frac{-1.03^\circ}{1 \times 0.970} = -1.06^\circ; [M]_D^{25} = -2.02^\circ$$

$$\text{Maximum } [M]_D^{25} = -3.47^\circ \text{ (homogeneous)}$$

Levo-5-Methylnonaneamide—50 gm. of 5-methylnonanoic chloride, $[M]_D^{25} = -2.02^\circ$ (homogeneous), were dropped into 200 cc. of ammonia as previously described. The amide was purified by crystallization.

$$[\alpha]_D^{25} = \frac{-0.20^\circ \times 100}{1 \times 20 \times 1.90} = -0.53^\circ; [M]_D^{25} = -0.91^\circ$$

$$\text{Maximum } [M]_D^{25} = -1.6^\circ \text{ (75\% alcohol)}$$

Levo-5-Methylnonanenitrile—21 gm. of 1-bromo-4-methyloctane,

$$[\alpha]_D^{25} = \frac{-2.20^\circ}{1 \times 1.09} = -2.02^\circ \text{ (homogeneous)}$$

were treated with KCN as usual. Yield of nitrile 12.5 gm. B.p. $96-102^\circ$, $p = 12$ mm. $d_4^{25} = 0.8187$ (*in vacuo*). $n_D^{25} = 1.4290$.

$$[\alpha]_D^{25} = \frac{-1.43^\circ}{1 \times 0.819} = -1.75^\circ; [M]_D^{25} = -2.68^\circ$$

$$\text{Maximum } [M]_D^{25} = -5.32^\circ \text{ (homogeneous)}$$

$$[\alpha]_{5875.6}^{25} = \frac{-1.951^{\circ} \times 100}{10 \times 10.92} = -1.79^{\circ}; \quad [M]_{5875.6}^{25} = -2.74^{\circ}$$

$$\text{Maximum } [M]_{5875.6}^{25} = -5.44^{\circ} \text{ (heptane)}$$

4.695 mg. substance: 0.383 cc. N_2 at 20° and 765 mm.

$C_{10}H_{19}N$ (153.2). Calculated, N 9.1; found, N 9.4

Levo-1-Bromo-5-Methylnonane—45 gm. of 5-methylnonanol-1, $\alpha = 0$, from 5-methylnonanoic acid, $[M]_D^{25} = -0.99^{\circ}$ (homogeneous), were brominated with 100 gm. of phosphorus tribromide as usual. B.p. 110° at 15 mm. Yield 50 gm. $d_4^{25} = 1.062$.

$$[\alpha]_D^{25} = \frac{-1.08^{\circ}}{1 \times 1.062} = -1.02^{\circ}; \quad [M]_D^{25} = -2.26^{\circ}$$

$$\text{Maximum } [M]_D^{25} = -5.3^{\circ} \text{ (homogeneous)}$$

6.900 mg. substance: 13.770 mg. CO_2 and 5.765 mg. H_2O

7.545 " " : 6.450 " silver bromide

$C_{10}H_{21}Br$. Calculated. C 54.3, H 9.6, Br 36.2

221.1 Found. " 54.4, " 9.4, " 36.4

Levo-6-Methyldecanenitrile—17 gm. of 1-bromo-5-methylnonane,

$$[\alpha]_D^{25} = \frac{-1.30^{\circ}}{1 \times 1.062} = -1.22^{\circ} \text{ (homogeneous)}$$

when refluxed with KCN in 80 per cent alcohol, yielded 9 gm. of nitrile. B.p. $116-118^{\circ}$, $p = 15$ mm. $d_4^{25} = 0.8207$ (*in vacuo*). $n_D^{25} = 1.4319$.

$$[\alpha]_D^{25} = \frac{-0.49^{\circ}}{1 \times 0.821} = -0.60^{\circ}; \quad [M]_D^{25} = -1.00^{\circ}$$

$$\text{Maximum } [M]_D^{25} = -1.95^{\circ} \text{ (homogeneous)}$$

$$[\alpha]_{5875.6}^{25} = \frac{-0.769^{\circ} \times 100}{10 \times 12.72} = -0.60^{\circ}; \quad [M]_{5875.6}^{25} = -1.00^{\circ}$$

$$\text{Maximum } [M]_{5875.6}^{25} = -1.95^{\circ} \text{ (heptane)}$$

3.210 mg. substance: 9.340 mg. CO_2 and 3.710 mg. H_2O

$C_{11}H_{21}N$. Calculated. C 78.95, H 12.67

167.2 Found. " 79.34, " 12.93

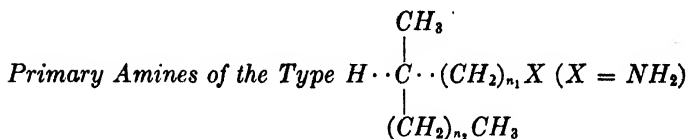
OPTICAL ROTATIONS IN HOMOLOGOUS SERIES OF ALIPHATIC AMINES

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The conduct of amines of the above type was analyzed with the object of comparing the partial rotation of the amino group with that of the hydroxyl group in substances differing structurally only in X (X = NH₂ or OH).

The configurational relationship of the amines to be discussed here was established by the mode of their preparation either from the nitriles or from the bromides of known configuration.

In Table I the rotations of the homologous series of amines are compared with those of corresponding carbinols. It may be mentioned here that the amines given in the third column of Table I were analyzed in greater detail, for the reason that more material was available. Besides, all the substances of this series were prepared independently by two workers. There are recorded many observations tending to show that the amino group, with respect to its partial rotation, functions very similarly to a hydroxyl group. In primary aliphatic carbinols the changes of rotation with the increase of the values of n_1 and of n_2 are very significant; the question is whether a similar state of affairs can be observed in the corresponding amines. The answer to the question is given in Table I.

Three important conclusions emerge from the observations recorded in Tables I and II.

TABLE I
Configurally Related Aliphatic Amines. $[M]_D^{25}$ Maximum (Homogeneous)

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ -5.1^\circ* \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +11.1^\circ\dagger \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +12^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +16^\circ\dagger \end{array}$	
Carbinol‡	+5.2°	+12°	+12°	
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ -14^\circ§ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ -0.4^\circ\parallel \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{NH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \\ -0.7^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{NH}_2 \\ \\ \text{C}_2\text{H}_7 (n) \end{array}$	
Carbinol‡	-6.8°	-2.1°	0	+1.7°
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_9 (n) \\ -16^\circ\P \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_9 (n) \\ -1.7^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{NH}_2 \\ \\ \text{C}_2\text{H}_9 (n) \\ -0.8^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{NH}_2 \\ \\ \text{C}_2\text{H}_9 (n) \\ +0.8^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_5\text{NH}_2 \\ \\ \text{C}_2\text{H}_9 (n) \\ +2.4^\circ \end{array}$
Carbinol‡	-7.9°	-4.0°	-0.7°	0

* Marckwald, W., *Ber. chem. Ges.*, **37**, 1045 (1904).

† In dilute alcohol.

‡ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

§ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 577 (1929).

|| Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 92 (1930).

¶ In ether.

TABLE II
Configurally Related Hydrochlorides of Amines. $[M]_D^{25}$ Maximum

CH_3 $\text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2\text{HCl}$ C_2H_5 -0.2^{**}	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{NH}_2\text{HCl}$ C_2H_5 $+9.9^{**}$	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{NH}_2\text{HCl}$ C_2H_5 $+13.0^{**}$	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{NH}_2\text{HCl}$ C_2H_5 $+16.0^{\dagger}$
CH_3 $\text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2\text{HCl}$ $\text{C}_4\text{H}_9 (n)$ -18.0^{\dagger}	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{NH}_2\text{HCl}$ $\text{C}_4\text{H}_9 (n)$ $+2.1^{\dagger}$	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{NH}_2\text{HCl}$ $\text{C}_4\text{H}_9 (n)$ -0.6^{\dagger}	CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_4\text{NH}_2\text{HCl}$ $\text{C}_4\text{H}_9 (n)$ $+1.2^{\dagger}$
			CH_3 $\text{H} \cdots \text{C} \cdots (\text{CH}_2)_5\text{NH}_2\text{HCl}$ $\text{C}_4\text{H}_9 (n)$ $+3.5^{**}$

* In water.

† In dilute alcohol.

‡ In methanol.

1. The maximum rotations of the corresponding members of the two groups of substances are of the same order of magnitude.

2. The molecular rotations decline with the progressive increase in the value of n_1 and the sign of rotation changes when $n_1 = n_2 + 1$. (The 0 values in the case of carbinols do not indicate loss of asymmetry, inasmuch as the same carbinols on bromination form optically active bromides which, in turn, lead to optically active carbinols.) Hence, in the case of the primary amines, the NH_2 group functions similarly to an OH group.

3. In Table II are given the rotations of the hydrochlorides of the bases containing an ethyl or a normal butyl group. From Table II it can be seen that with the progressive increase in the value of n_1 , there occurs a periodic change in the direction of the shift of rotation.

Thus, the free amines recorded in Table I function (with respect to their optical activity) similarly to the carbinols, whereas in ionized state their behavior resembles closely that of the acids and of the aldehydes.

EXPERIMENTAL

*Dextro-1-Amino-3-Methylpentane*¹—9.5 gm. of 3-methylpentane-nitrile, $[\alpha]_D^{25} = +1.97^\circ$ (homogeneous), were dissolved in methanol, Raney's catalyst was added, and the solution was shaken with hydrogen at atmospheric pressure for 2 days. The catalyst was filtered off, and hydrogen chloride in methanol was added to the filtrate. This was evaporated to dryness with benzene. The crystals were taken up in petroleum ether and filtered. Yield 10 gm.

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.21^\circ \times 100}{4 \times 3.33} = +1.58^\circ; \quad [M]_D^{25} = +2.17^\circ$$

$$\text{Maximum } [M]_D^{25} = +9.85^\circ \text{ (hydrochloride in H}_2\text{O)}$$

$$[\alpha]_D^{25} = \frac{+0.13^\circ \times 100}{4 \times 1.36} = +2.39^\circ; \quad [M]_D^{25} = +2.41^\circ$$

$$\text{Maximum } [M]_D^{25} = +10.9^\circ \text{ (free amine in 44 \% alcohol)}$$

5.790 mg. substance: 0.509 cc. N_2 at 24° and 757.5 mm.

$\text{C}_6\text{H}_{15}\text{NCl}$ (137.6). Calculated, N 10.18; found, N 10.07

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

Dextro-1-Amino-4-Methylhexane—13 gm. of 4-methylhexane-nitrile, $[\alpha]_D^{25} = +5.71^\circ$ (homogeneous), were dissolved in methanol and reduced with hydrogen in the presence of Raney's catalyst. About 3.5 liters of hydrogen were absorbed. The catalyst was then filtered off. Hydrogen chloride in methanol was added to the filtrate, and this was then concentrated to dryness under reduced pressure. The residue was dissolved in benzene and again concentrated to dryness. The crystals were taken up in pentane and filtered. Yield 17 gm.

4.801 mg. substance: 0.392 cc. N_2 at 24° and 751 mm.

4.095 " " : 8.300 mg. CO_2 and 4.300 mg. H_2O

$C_7H_{15}NCl$. Calculated. C 55.41, H 11.96, N 9.24

151.6 Found. " 55.27, " 11.75, " 9.27

The hydrochloride was dissolved in 50 per cent NaOH, and the free amine was extracted with ether. The extract was dried over metallic sodium. The amine distilled at 80° , $p = 90$ mm. $d_4^{25} = 0.7709$ (in vacuo). $n_D^{25} = 1.4220$.

$$[\alpha]_{5875.6}^{25} = \frac{+5.755^\circ}{2 \times 0.771} = +3.73^\circ; \quad [M]_{5875.6}^{25} = +4.30^\circ$$

Maximum $[M]_{5875.6}^{25} = +11.7^\circ$ (homogeneous)

3.2524 gm. of the amine were neutralized with 1 equivalent of HCl and diluted to 20 cc. with water.

$$[\alpha]_{5875.6}^{25} = \frac{+2.75^\circ \times 100}{4 \times 21.42} = +3.21^\circ; \quad [M]_{5875.6}^{25} = +4.87^\circ$$

Maximum $[M]_{5875.6}^{25} = +13.2^\circ$ (hydrochloride in H_2O)

Dextro-1-Amino-5-Methylheptane—10 gm. of 5-methylheptane-nitrile, $[\alpha]_D^{25} = +4.51^\circ$ (homogeneous), were dissolved in methanol, and Raney's catalyst was added. This was shaken in 1 atmosphere of hydrogen for 8 hours. The amine hydrochloride was isolated as described above. The rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.41^\circ \times 100}{4 \times 3.33} = +3.08^\circ; \quad [M]_D^{25} = +5.10^\circ$$

Maximum $[M]_D^{25} = +15.7^\circ$ (hydrochloride in 33% alcohol)

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$$[\alpha]_D^{25} = \frac{+0.25^\circ \times 100}{4 \times 1.55} = +4.03^\circ; \quad [M]_D^{25} = +5.20^\circ$$

Maximum $[M]_D^{25} = +16.0^\circ$ (amine in 60% alcohol)

4.910 mg. substance: 10.475 mg. CO₂ and 5.290 mg. H₂O

6.580 " " : 0.481 cc. N₂ at 22° and 771 mm.

C₈H₂₀NCl. Calculated. C 58.0, H 12.2, N 8.5

165.6 Found. " 58.2, " 12.1, " 8.6

Dextro-1-Amino-4-Methylheptane—15 gm. of 4-methylheptanenitrile, $[M]_D^{25} = -0.50^\circ$, were reduced by 60 gm. of sodium in 600 cc. of absolute alcohol. The amine was purified through its hydrochloride. B.p. 165° at 760 mm. $d_4^{25} = 0.784$.

$$[\alpha]_D^{25} = \frac{+0.20^\circ}{2 \times 0.784} = +0.13^\circ; \quad [M]_D^{25} = +0.17^\circ$$

Maximum $[M]_D^{25} = +0.68^\circ$ (homogeneous)

0.0994 gm. substance: 7.65 cc. 0.1 N hydrochloric acid

C₈H₁₉N (129.15). Calculated, N 10.8; found, N 10.7

Levo-1-Amino-2-Methylhexane—5 gm. of 2-methylhexanenitrile, $[\alpha]_D^{25} = +9.05^\circ$ (homogeneous), were dissolved in methanol and shaken with hydrogen at atmospheric pressure, in the presence of Raney's catalyst, for 24 hours. The amine hydrochloride was isolated as usual.

$$[\alpha]_{5875.6}^{25} = \frac{-0.48^\circ \times 100}{4 \times 5.00} = -2.40^\circ; \quad [M]_{5875.6}^{25} = -3.64^\circ$$

Maximum $[M]_{5875.6}^{25} = -18.2^\circ$ (hydrochloride in methanol)

$$\text{Free amine}^2 \frac{3.63}{4.03} \times 18.2 = -16.4^\circ \text{ (amine in ether)}$$

5.805 mg. substance: 0.472 cc. N₂ at 28° and 763 mm.

C₇H₁₅NCl (151.6). Calculated, N 9.24; found, N 9.25

Levo-1-Amino-3-Methylheptane—3-Methylheptanenitrile, $[\alpha]_D^{25} = +2.96^\circ$ (homogeneous), were reduced with Raney's catalyst in methanol with hydrogen at atmospheric pressure as usual. The hydrochloride had a rotation of

² Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 583 (1929).

$$[\alpha]_{5875.6}^{25} = \frac{+0.16^\circ \times 100}{4 \times 5.00} = +0.80^\circ; \quad [M]_{5875.6}^{25} = +1.32^\circ$$

Maximum $[M]_{5875.6}^{25} = +2.13^\circ$ (hydrochloride in methanol)

The free amine was isolated from the salt as usual. B.p. 65–66°, = 14 mm.

$$[\alpha]_D^{25} = \frac{-0.65^\circ}{1 \times 0.782} = -0.83^\circ; \quad [M]_D^{25} = -1.07^\circ$$

Maximum $[M]_D^{25} = -1.72^\circ$ (homogeneous)

4.370 mg. substance: 11.900 mg. CO₂ and 5.750 mg. H₂O

C₈H₁₉N. Calculated. C 74.31, H 14.85

129.2 Found. " 74.26, " 14.72

Levo-1-Amino-4-Methyloctane—7 gm. of 4-methyloctanenitrile, $[\alpha]_D^{25} = -1.04^\circ$ (homogeneous), were reduced with hydrogen in the presence of Raney's catalyst, as described above. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{-0.13^\circ \times 100}{2 \times 20.0} = -0.33^\circ; \quad [M]_D^{25} = -0.59^\circ$$

Maximum $[M]_D^{25} = -0.63^\circ$ (hydrochloride in methanol)

The free base was isolated as usual. B.p. 75–77°, $p = 13$ mm. $d_4^{25} = 0.780$.

$$[\alpha]_D^{25} = \frac{-0.40^\circ}{1 \times 0.780} = -0.51^\circ; \quad [M]_D^{25} = -0.73^\circ$$

Maximum $[M]_D^{25} = -0.78^\circ$ (homogeneous)

4.420 mg. substance: 12.305 mg. CO₂ and 5.870 mg. H₂O

C₉H₂₁N. Calculated. C 75.43, H 14.79

143.2 Found. " 75.46, " 14.86

Levo-1-Amino-5-Methylnonane—10 gm. of 5-methylnonanenitrile, $[\alpha]_D^{25} = -1.75^\circ$ (homogeneous), were dissolved in methanol and reduced with hydrogen in the presence of Raney's catalyst. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{-0.12^\circ \times 100}{4 \times 10.0} = -0.30^\circ; \quad [M]_D^{25} = -0.58^\circ$$

Maximum $[M]_D^{25} = -1.15^\circ$ (hydrochloride in methanol)

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The free base was liberated and distilled. B.p. 92° at 15 mm.
Yield 10 gm. $d_4^{25} = 0.799$.

$$[\alpha]_D^{25} = \frac{-0.19^{\circ}}{1 \times 0.799} = -0.24^{\circ}; \quad [M]_D^{25} = -0.38^{\circ}$$

$$\text{Maximum } [M]_D^{25} = -0.75^{\circ} \text{ (homogeneous)}$$

34.48 mg. substance: 5.39 cc. N_2 at 23° and 763 mm.

$C_{10}H_{23}N$ (157.2). Calculated, N 8.91; found, N 8.86

Levo-1-Amino-6-Methyldecane—20 gm. of 6-methyldecanenitrile, $[M]_D^{25} = -0.94^{\circ}$ (homogeneous), were reduced with 40 gm. of sodium in absolute alcohol. After purification through its hydrochloride, the amine boiled at 113° , $p = 15$ mm. Yield 19 gm. $d_4^{22} = 0.798$.

$$[\alpha]_D^{25} = \frac{-0.49^{\circ}}{1 \times 0.798} = -0.61^{\circ}; \quad [M]_D^{25} = -1.04^{\circ}$$

$$\text{Maximum } [M]_D^{25} = -2.44^{\circ} \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{-0.13^{\circ} \times 100}{2 \times 9.094} = -0.71^{\circ}; \quad [M]_D^{25} = -1.48^{\circ}$$

$$\text{Maximum } [M]_D^{25} = -3.47^{\circ} \text{ (hydrochloride in water)}$$

3.710 mg. substance: 10.540 mg. CO_2 and 4.745 mg. H_2O
20.000 " " : 2.95 cc. N_2 at 25° and 763 mm.

$C_{11}H_{25}N$. Calculated. C 77.1, H 14.7, N 8.2
171.2 Found. " 77.5, " 14.3, " 8.2

NOTE ON THE HYDROGENATION OF PHENYLATED CARBINOLS

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(Received for publication, May 29, 1936)

On the occasion of hydrogenation of phenylated carbinols previously reported¹ the predominating product was a hydrocarbon, the cyclohexylcarbinol constituting by far the smaller portion, so that its purification required great care. Inasmuch as the hydrogenation then undertaken had for its object the comparison of the rotations of the cyclohexyl and of the phenyl derivatives, the question of the purity of the former was of great importance. It was therefore desirable to seek conditions under which the formation of the hydrocarbon could be avoided. Change in the nature of the catalyst did not lead to the desired result. Change in the solvent, however, did. Thus in a solution of methanol Adams' catalyst fails to affect the hydrogenation of the benzene ring. However, in methanol containing 10 per cent of glacial acetic acid the reaction stops at the formation of the cyclohexylcarbinol, the fraction of the hydrocarbon being minimal.

This selective action of the solvent was found to be helpful when it was desired to hydrogenate the side chain, leaving the benzene nucleus unchanged. Thus phenylated azides and phenylated nitriles are readily reduced to the amines, even under pressure, leaving the benzene nucleus intact.

The relationships of the rotations of the phenylated and cyclohexylcarbinols reported by Levene and Stevens² are substantiated by the present experiments.

¹ Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **89**, 471 (1930).

² Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **87**, 375 (1930).

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EXPERIMENTAL

*Reduction in Methanol and Acetic Acid*²—12 gm. of methylphenylcarbinol,

$$[\alpha]_D^{25} = \frac{+7.20^\circ}{1 \times 1.008} = +7.14^\circ \text{ (homogeneous)}$$

were dissolved in 25 cc. of a solution containing 90 gm. of methanol and 10 gm. of glacial acetic acid. 0.5 gm. of Adams' catalyst was added and the mixture was reduced with hydrogen under a pressure of 45 pounds for 16 hours. The catalyst was filtered off, and the filtrate was distilled at atmospheric pressure to remove the methanol. The residue was taken up in ether and washed with potassium carbonate solution. The extract was dried over anhydrous potassium carbonate. The carbinol was then distilled. Yield 6.5 gm. B.p. 77–78°, $p = 12$ mm.; and 112°, $p = 55$ mm. $n_D^{25} = 1.4643$.

$$[\alpha]_D^{25} = \frac{-0.83^\circ}{1 \times 0.908} = -0.91^\circ \text{ (homogeneous)}$$

The substance analyzed as follows:

4.525 mg. substance: 12.480 mg. CO₂ and 5.090 mg. H₂O
 C₈H₁₀O. Calculated. C 74.92, H 12.59
 128.1 Found. " 75.21, " 12.58

In another experiment 10 gm. of inactive carbinol, when shaken with 0.5 gm. of catalyst in 25 cc. of the solution for 48 hours, gave a pressure change of 24 pounds.³ The yield of carbinol was 5 gm.

*Reduction in Glacial Acetic Acid*¹—10 gm. of inactive methylphenylcarbinol were dissolved in 25 cc. of glacial acetic acid. 0.5 gm. of Adams' catalyst was added, and the mixture was shaken in the presence of hydrogen at an initial pressure of 45 pounds for 16 hours. The final pressure was 21 pounds. The substance was isolated as usual. Fraction I, b.p. 133–140°, $p =$ atmospheric (ethyl benzene fraction); Fraction II, b.p. 86–87°, $p = 13$ mm. Weight 3.5 gm. $n_D^{25} = 1.4644$.

4.398 mg. substance: 12.100 mg. CO₂ and 4.935 mg. H₂O
 C₈H₁₀O. Calculated. C 74.92, H 12.59
 128.1 Found. " 75.02, " 12.55

² A Burgess-Parr Illinois model hydrogenation apparatus was used for all the experiments.

In another experiment 10 gm. of carbinol with 0.7 gm. of catalyst in 25 cc. of glacial acetic acid, when shaken for 40 hours, gave a pressure change of 29 pounds. The yield of carbinol was only 1 gm.

Reduction in Methanol—10 gm. of inactive methylphenylcarbinol were dissolved in 25 cc. of methanol, and 0.5 gm. of Adams' catalyst was added. This was shaken in the presence of hydrogen at a pressure of 47 pounds for 16 hours. The pressure at the end of this time was 46 pounds. The mixture was filtered and the filtrate distilled at atmospheric pressure to remove the methanol. The carbinol was distilled. B.p. 88–89°, $p = 13$ mm. Weight 8.5 gm. $n_D^{25} = 1.5250$.

4.908 mg. substance: 14.160 mg. CO₂ and 3.690 mg. H₂O

C₈H₁₀O. Calculated. C 78.64, H 8.27

122.1 Found. " 78.67, " 8.40

In an identical experiment, shaking for 48 hours produced the same result.

THE RELATION OF THE ADRENAL MEDULLA TO THE EFFECT OF INSULIN ON PURINE METABOLISM

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In previous communications it was shown that the subcutaneous injection of insulin led to an increased excretion of allantoin in the ordinary breed of dog (1), whereas in the Dalmatian coach-dog this hormone effected an increased excretion of uric acid (2). In the latter breed, the increased excretion of uric acid in the urine accompanied a rise in the blood uric acid level. That the rise in the blood uric acid was secondary to the hypoglycemia rather than a direct effect of insulin was shown to be the case when it was observed that this rise did not occur if glucose sufficient to prevent the fall in blood sugar was administered prior to the injection of insulin. Since hypoglycemia may be a sufficient stimulus to evoke an increased secretion of epinephrine from the adrenal gland, the hypothesis was entertained that it was by way of epinephrine that insulin affected the purine metabolism. In a third communication, Chaikoff, Larson, and Read succeeded in showing that the subcutaneous injection of epinephrine increased the excretion of allantoin in the ordinary breed of dog as well as the excretion of both allantoin and uric acid in the Dalmatian coach-dog (3). Again, in the latter breed, the increased excretion of uric acid in the urine accompanied a rise in the blood uric acid level. It therefore seemed entirely likely that epinephrine was the direct stimulus to the increased excretion of allantoin and uric acid observed under the influence of insulin.

Definite proof for the proposed mechanism for the effect of insulin on purine metabolism necessitates observations on the effect of insulin on purine metabolism in the absence of the adrenal medulla. To supply these has been the purpose of the present investigation.

EXPERIMENTAL

Operative Procedure—Female dogs were used in these experiments. Operative removal of the adrenal medulla was performed in two stages, either the right or left adrenal being removed at one operation and after complete healing and good scar formation adrenalectomy of the opposite side was performed. A small segment of each gland containing no apparent medullary tissue was left intact. Postoperative care was concentrated on keeping the animals warm at all times and on preventing wound infections or healing them if they occurred.

Dog P—Partial adrenalectomy on the left side was performed on December 16, 1935, a small cortical remnant of the lower pole of the gland being left intact. On January 16, 1936, partial adrenalectomy on the right side was accomplished and a long longitudinal strip of cortical tissue was left which showed no admixture of the dark medullary substance. Recovery was rapid and the animal was placed on a special diet, as described below, January, 25, 1936.

Subsequent to the experiment an autopsy was performed on March 17, 1936. There were no gross pathological lesions. Study of tissues in close relation to the site of adrenalectomy on the right side showed no remaining adrenal tissue. On the left side, there was grossly one small nodule of glandular tissue. Serial sections through this nodule showed a small island of medullary tissue imbedded in cortical cells. It was estimated that the amount of remaining medullary tissue represented about 3 to 5 per cent of the medullary portion of the intact gland.

Dog V—Right partial adrenalectomy was performed on January 29, 1936. In this operation two slices of cortex were left behind, one from the upper and one from the lower pole of the gland. Following the left partial adrenalectomy on March 3, 1936, during which a portion of the lower pole was left intact, the subsequent recovery of the animal was uneventful.

After completion of the experiment, an autopsy was performed on April 27, 1936. Histological examination of the remaining adrenal tissue showed on the left side one fragment of glandular tissue containing about 3 per cent of the amount of medullary substance ordinarily found in the entire gland. The remaining glandular tissue on the right side revealed approximately the same proportion of medulla as found on the left side.

No separate tests were conducted to determine the activity of the remaining medullary tissue found at autopsy. However, in regard to the present experiment, it possessed no apparent physiological activity.

Care of Animals—Following postoperative recovery the animals were kept in metal metabolism cages and fed a synthetic diet devised by Cowgill (4). The diet, which included Karr's salt mixture (5), was thoroughly mixed in quantities sufficient for each complete experiment. The uniformity of the mixture was tested by nitrogen determinations on random samples. Fat, consisting of 17 gm. of lard and 9 gm. of unsalted butter per 100 gm. of the above mixture, was added at each feeding. Each dog received about 70 calories per kilo of body weight and maintained a good appetite throughout the experiment. 10 days of feeding sufficed to bring the animals into nitrogen equilibrium. 4 days of an equilibrium in which the daily excretion of nitrogen did not vary by more than 0.20 gm. were accepted as a suitable control period prior to the injection of insulin.

The dogs were fed once each day and catheterized twice daily. The time intervals of catheterization were planned with respect to the time of feeding so that samples of urine were obtained for the 5 hour interval prior to feeding and for the 19 hour interval after the ingestion of food.

All injections of insulin and epinephrine were made subcutaneously. With one exception, the insulin dosage employed was 0.5 unit per kilo of body weight, a dose which has been shown to affect the purine metabolism of the normal dog (1). In Dog V it was deemed advisable to give a slightly smaller dose at the time of the second injection, inasmuch as 0.5 unit per kilo resulted in noticeable symptoms of hypoglycemia on the date of the first injection. It may be said that both animals showed a much greater sensitivity to insulin than does the normal dog. The doses of epinephrine used were ones which, when given to the dog in this manner, have been shown to be insufficient to raise the blood pressure (6).

Methods

The following analytical procedures for urine were employed: total nitrogen, Kjeldahl method; allantoin, the method of Read and Chaikoff (7).

TABLE I

Effect of Insulin and Epinephrine on Allantoin Excretion of Partially Adrenalectomized Dogs

	Date	Allantoin N			Total N		
		19 hrs.	5 hrs.	24 hrs.	19 hrs.	5 hrs.	24 hrs.
		gm.	gm.	gm.	gm.	gm.	gm.
Dog P	Feb. 4	0.173	0.048	0.221	3.74	0.56	4.30
	" 5	0.164	0.046	0.210	3.58	0.57	4.15
	" 6	0.169	0.046	0.215	3.72	0.66	4.38
	" 7	0.174	0.048	0.222	3.60	0.65	4.25
	" 8	0.170	0.050	0.220	3.61	0.86	4.47
	" 9	0.167	0.045	0.212	3.50	0.58	4.08
	" 10	0.169	0.071	0.240	3.64	0.88	4.52
	" 11	0.193	0.048	0.241	3.45	0.58	4.03
	" 12	0.166	0.045	0.211	3.60	0.62	4.22
	" 13	0.174	0.045	0.219	3.58	0.94	4.52
	" 14	0.168			3.40		
Dog V	Apr. 8	0.110	0.024	0.134	3.57	0.49	4.06
	" 9	0.111	0.022	0.133	3.64	0.49	4.06
	" 15	0.115	0.020	0.135	3.65	0.44	4.09
	" 16	0.113	0.022	0.135	3.49	0.75	4.24
	" 17	0.116	0.023	0.139	3.50	0.45	3.95
	" 18	0.114	0.058	0.172	3.54	0.65	4.19
	" 19	0.122	0.021	0.143	3.59	0.43	4.02
	" 20	0.112	0.024	0.136	3.60	0.45	4.05
	" 21	0.115	0.022	0.137	3.52	0.61	4.13
	" 22	0.114	0.021	0.135	3.49	0.46	3.95

Dog P—This dog was catheterized at 8.45 a.m. and 1.45 p.m. daily; fed at 1.50 p.m. daily. Diet: 90 gm. of diet mixture plus 15 gm. of lard plus 8 gm. of unsalted butter. Diet N = 5.45 gm. per 100 gm. of diet mixture. On February 8, 4 units of insulin were injected subcutaneously at 8.50 a.m. On February 10, 0.8 mg. of epinephrine was injected subcutaneously at 8.50 a.m. On February 13, 4 units of insulin were injected subcutaneously at 8.50 a.m. The period of 19 hours represents the interval from 1.45 p.m. to 8.45 a.m.; the period of 5 hours, from 8.45 a.m. to 1.45 p.m. The dog weighed 7.9 kilos.

Dog V—This dog was catheterized at 10.00 a.m. and 3.00 p.m. daily; fed at 3.05 p.m. daily. Diet: 85 gm. of diet mixture plus 14 gm. of lard plus 7 gm. of unsalted butter. Diet N = 5.53 gm. per 100 gm. of diet mixture. On April 16, 3.5 units of insulin were injected subcutaneously at 10.05 a.m. On April 18, 1.0 mg. of epinephrine was injected subcutaneously at 10.05 a.m. On April 21, 2.5 units of insulin were injected subcutaneously at 10.05 a.m. The period of 19 hours represents the interval from 3.00 p.m. to 10.00 a.m.; the period of 5 hours, from 10.00 a.m. to 3.00 p.m. The dog weighed 7.0 kilos.

Results

The effect of insulin and epinephrine on the excretion of allantoin in two partially adrenalectomized dogs is shown in Table I. Essentially the same results were observed in both animals.

Dog P—The 5 hour excretion of allantoin nitrogen on the 4 days prior to the first injection of insulin remained quite constant, varying from 46 to 48 mg. Following the injection of 4 units of insulin there was no increase in the elimination of allantoin nor was the amount of allantoin excreted during the next 19 hours increased. However, when 0.8 mg. of epinephrine was injected, the allantoin nitrogen excreted for the 5 hours thereafter rose to 71 mg., an increase of 48 per cent, and the excretion for the subsequent 19 hours showed an increase of some 20 mg. above the control level. A second injection of 4 units of insulin led to no change in the allantoin excretion either in the 5 hour period thereafter or in the succeeding 19 hour interval.

Dog V—In this animal essentially the same results were obtained as in Dog P. On two occasions the injection of insulin led to no increase in the allantoin excretion, whereas when 1.0 mg. of epinephrine was injected, the allantoin excretion for the 5 hour period thereafter showed an increase of 140 per cent above the control level.

It may be noted that the daily excretion of allantoin nitrogen by Dog P was unusually high. In our experience, on only one previous occasion has a similar level of allantoin elimination been observed in the dog. However, this high rate of allantoin excretion did not prevent the establishment of a constant daily allantoin balance and in no wise affected the objects of this experiment.

SUMMARY

Insulin, administered subcutaneously in amounts that produce a marked increase in the allantoin excretion by normal dogs (1), has no effect on the allantoin output of dogs from which the adrenal medulla has been removed. The same marked increase in purine excretion which followed the injection of epinephrine in the normal dog (3) has been shown to follow the injection of this hormone in adrenalectomized animals.

We conclude:

1. That insulin *per se* has no effect on purine metabolism.

2. That epinephrine can accelerate the rate of purine metabolism in the adrenalectomized dog.

3. That the increased purine elimination observed to follow the subcutaneous injection of insulin in the intact dog (1, 2) is dependent upon the ability of insulin hypoglycemia to effect an increased secretion of epinephrine from the adrenal medulla.

We wish to express our gratitude to Professor James F. O'Donnell of the Department of Anatomy for the necropsy studies of our material.

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DERIVATIVES OF GLUCURONIC ACID

VII. THE SYNTHESIS OF ALDOBIONIC ACIDS*

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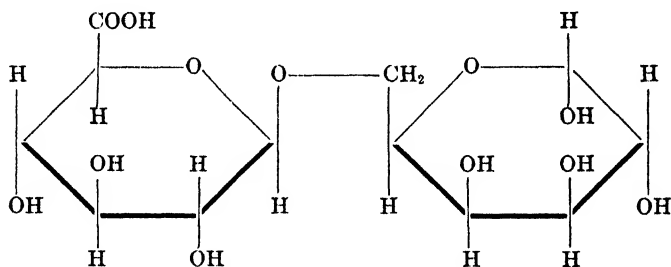
In Paper VI of this series, the preparation of α -bromotriacetylglucuronic acid methyl ester was described (1). It was shown that when the latter derivative is condensed with alcohols in the presence of silver oxide, the corresponding β -glucuronides are formed. The suggestion was made that the successful preparation of acetobromoglucuronic ester should make possible the synthesis not only of glucuronides, but of aldobionic acids as well.

Aldobionic acids may be defined as disaccharides containing a uronic acid as one of the sugar components. These sugar acids were first found among the hydrolysis products of the type-specific polysaccharides of certain encapsulated bacteria (2). Aldobionic acids have since been obtained from a variety of plant gums. Thus, when gum acacia is hydrolyzed with dilute mineral acid, there is obtained an aldobionic acid, galactose glucuronide (3, 4). Challinor, Haworth, and Hirst (5) have shown this substance to have the structure of a galactopyranose-6-glucuronopyranoside and have suggested that the biose linkage possesses the β configuration. The present communication describes the chemical synthesis of this aldobionic acid, and of a second aldobionic acid, the heptaacetyl methyl ester of glucose-6- β -glucuronide.

By using a reaction similar to that employed by Freudenberg, Noë, and Knopf in synthesizing the disaccharide galactose-6- β -glucoside (6), it has been possible to prepare synthetically an aldobionic acid identical with that obtained from gum acacia. When

* For preliminary communications see *Science*, **83**, 353 (1936); *J. Am. Chem. Soc.*, **58**, 858 (1936).

1,2,3,4-diacetonegalactose is condensed with α -bromotriacetylglucuronic acid methyl ester in ether solution in the presence of silver oxide, diacetonegalactose-6- β -triacetylglucuronide methyl ester is produced. Upon saponification with barium hydroxide and subsequent removal of the acetone groups by boiling with dilute sulfuric acid, the crystalline aldobionic acid is obtained. The synthetic acid is identical in properties with the naturally occurring aldobionic acid obtained from gum acacia, and the melting point of a mixture of the two substances shows no depression. Unless inversion has occurred, the synthetic acid must possess the β -biose configuration, inasmuch as the acetobromo derivative of glucuronic acid from which it is prepared has been found to yield only β -glucuronides (1). It is therefore possible to confirm the suggestion of Challinor, Haworth, and Hirst that the aldobionic acid of gum acacia has the β configuration, as shown in Formula I.



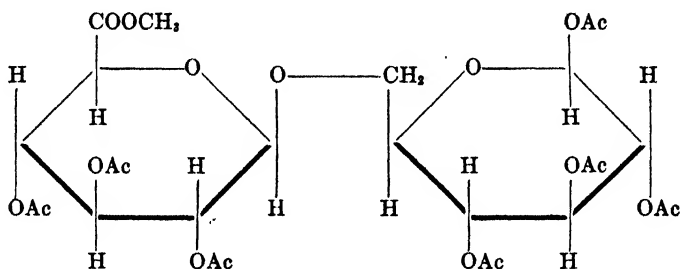
I

Further confirmation of the identity of the synthetic and natural aldobionic acids was afforded by the preparation of the corresponding heptaacetyl methyl esters. In each case the aldobionic acid was converted into the methyl ester by the action of diazomethane, followed by acetylation with acetic anhydride and pyridine. There was obtained, from both the synthetic and the natural acids, the same crystalline heptaacetyl methyl ester, possessing well defined and identical physical properties.

The synthesis of the aldobionic acid of gum acacia, already shown by Challinor, Haworth, and Hirst to be a glucuronopyranoside, indicates that the acetobromo derivative of glucuronic acid methyl ester itself is a pyranoside. This bromo derivative may

therefore be assigned the structure 1-(α)-bromo-2,3,4-triacetylglucuronic acid methyl ester.

Disaccharides have been synthesized by Helferich and his co-workers by condensing 1,2,3,4-tetraacetyl- β -glucose with acetobromo sugars (7). The synthesis of the heptaacetyl methyl ester of the aldobionic acid, glucose-6- β -glucuronide, has been accomplished by condensing 1,2,3,4-tetraacetyl- β -glucose with 1-bromo-2,3,4-triacetylglucuronic acid methyl ester in chloroform solution in the presence of silver oxide. β -Heptaacetylglucose-6- β -glucuronide methyl ester, represented by Formula II, is obtained as a



II

crystalline substance with the specific rotation $[\alpha]_D = -11.0^\circ$. Inasmuch as the aldobionic acid glucose-6- β -glucuronide may be considered as derived from the disaccharide gentiobiose (glucose-6- β -glucoside), the name β -heptaacetylgentiobiuronic acid methyl ester is suggested for the synthetic derivative.

The synthetic β -heptaacetate of gentiobiuronic acid methyl ester has been converted into its α isomer by the action of zinc chloride in acetic anhydride solution. The difference in molecular rotation of the α and β forms in chloroform is $39,500^\circ$, a value in close agreement with the known differences in molecular rotation of the α and β isomers of sugar acetates.

EXPERIMENTAL

Synthesis of 1,2,3,4-Diacetonegalactose-6- β -2,3,4-Triacetylglucuronide Methyl Ester—To 4.4 gm. of diacetonegalactose (8), dissolved in 100 cc. of anhydrous ether, were added 3.5 gm. of dry silver oxide and 6.75 gm. of α -bromotriacetylglucuronic acid methyl ester (1). The mixture was shaken until the solution no

longer contained the free bromine derivative. After filtering, the solution was evaporated *in vacuo* to a syrup. The latter was dissolved in 35 cc. of ethyl alcohol and treated with 10 cc. of water. After several hours 3.6 gm. of colorless needles were obtained, which on recrystallization from 50 per cent ethyl alcohol yielded 3.0 gm. of pure diacetonegalactose triacetylglucuronide methyl ester melting at 112.5–114° (uncorrected). The derivative is easily soluble in acetone, ether, methyl and ethyl alcohols, and chloroform.

$$[\alpha]_D^{24} = -68.0^\circ \text{ in chloroform } (c = 2.0 \text{ per cent})$$

Analysis— $C_{17}H_{24}O_{10}(COOCH_3)(COCH_3)_2$

Calculated. C 52.05, H 6.30, OCH₃ 5.38, COCH₃ 22.4

Found. " 51.85, " 6.45, " 5.39, " 21.5

Preparation of the Synthetic Aldobionic Acid, Galactose-6-β-Glucuronide—2.5 gm. of diacetonegalactose triacetylglucuronide methyl ester, prepared as above, were dissolved in 100 cc. of acetone and treated with 42 cc. of 0.44 N (4.2 equivalents) of barium hydroxide solution. After the addition of 20 cc. of water, the solution was left at room temperature for 4 hours. The barium was precipitated with N sulfuric acid and the filtrate evaporated *in vacuo* to remove the acetone. The residue was taken up in 150 cc. of 0.02 N sulfuric acid and boiled for 1½ hours. After cooling, the sulfuric acid was removed quantitatively by precipitation with barium hydroxide, followed by centrifugation to remove barium sulfate. The supernatant liquid was extracted once with ether to remove traces of condensation products of acetone and then concentrated *in vacuo* to a syrup. The syrup became crystalline on stirring with a small amount of water at 0°. A total of 1.5 gm. (92 per cent of the theoretical amount) of colorless needles of the aldobionic acid hydrate were recovered. The substance melted at 118–120° (uncorrected) with effervescence. A sample of aldobionic acid prepared from gum acacia melted at 116–119° (uncorrected). A mixture of this natural acid with the synthetic product melted likewise at 116–119°.

$[\alpha]_D^{20} = +9.4^\circ$ in water (after 3 minutes), changing to a final value of -7.3° within 2 hours ($c = 1$ per cent). The natural aldobionic acid hydrate gives an initial specific rotation of $+10.5^\circ$ (after 2 minutes) and a final value of -7.8° (3). The hydrate lost 2 moles

of water of crystallization on drying at 100° to constant weight. A weighed sample of the dried aldobionic acid, when analyzed by the method of Willstätter and Schudel (9), utilized the theoretical quantity of standard iodine solution. Thus, 0.574 gm. of aldobionic acid reduced 6.31 cc. of 0.05 N iodine solution (calculated, 6.45 cc.).

When the synthetic aldobionic acid is converted to the heptaacetyl methyl ester by the method described below, the purified derivative thus obtained melts at $200\text{--}201^{\circ}$ (uncorrected). The latter resembles in appearance and solubility the same derivative obtained from the natural aldobionic acid. A mixture of the two substances melts at $200\text{--}201^{\circ}$. The identity of these derivatives seems, therefore, to be definitely established.

Preparation of the Methyl Ester of Galactose-6- β -Glucuronide—8.8 gm. of aldobionic acid hydrate, prepared from gum acacia, were dissolved in 300 cc. of absolute methyl alcohol and cooled to 0° . An ethereal solution of diazomethane, prepared from 18 gm. of N-nitrosomethylurea (10), was slowly added until a faint yellow color persisted. The solution was concentrated to small volume and placed in the ice box for several days. There were isolated 7.9 gm. of colorless needles. The derivative crystallizes from methyl alcohol with approximately 1 mole of solvent of crystallization. After several recrystallizations from methyl alcohol and drying to constant weight, the methyl aldobionate melted unsharply at 119° (uncorrected), with effervescence.

$[\alpha]_D^{26} = -2.9^{\circ}$ in water (after 6 minutes), changing to a final value of -9.1° ($c = 4.0$ per cent).

Analysis— $C_{11}H_{19}O_{10}(COOCH_3)$

Calculated. C 42.14, H 5.99, OCH_3 8.38

Found. " 42.52, " 6.33, " 8.52

Preparation of the Heptaacetyl Methyl Ester of Galactose-6- β -Glucuronide—2.0 gm. of crystalline methyl aldobionate were dissolved in a cold mixture of 10 cc. of dry pyridine and 6 cc. of acetic anhydride and left at 25° for $2\frac{1}{2}$ hours. The reaction mixture was then poured into 250 cc. of ice water, 30 cc. of chloroform were added, and the suspension was cautiously treated at 0° with sodium hydroxide solution until just neutral. The mixture was extracted three times with chloroform; the extracts were dried and

concentrated *in vacuo*. The pyridine solution remaining was evaporated with absolute ethyl alcohol three times. The residual syrup was dissolved in 30 cc. of absolute ethyl alcohol and allowed to stand at room temperature for 24 to 48 hours until crystallization was complete. 1.4 gm. of acetyl ester melting at 198–200° were obtained. After recrystallization from absolute ethyl alcohol, the heptaacetylgalactose-6- β -glucuronide methyl ester was obtained in the form of shining needles, melting sharply at 202–203° (uncorrected).

$$[\alpha]_D^{25} = -17.5^\circ \text{ in chloroform } (c = 3 \text{ per cent})$$

Analysis— $C_{11}H_{12}O_{10}(COOCH_3)(COCH_3)_7$

Calculated. C 48.8, H 5.46, OCH_3 4.67, $COCH_3$ 45.3

Found. " 48.9, " 5.77, " 4.73, " 44.7

From the value of the specific rotation of the crystalline heptaacetate and from the fact that the amorphous material remaining in the filtrate possessed a higher specific rotation, it may be concluded that the crystalline substance is probably the β -heptaacetate.

Preparation of β -Heptaacetylgentiobiuronic Acid Methyl Ester—

To 20 cc. of dry, alcohol-free chloroform were added 1.82 gm. of α -bromotriacetylglucuronic acid methyl ester (1), 1.83 gm. of 1,2,3,4-tetraacetyl- β -glucose (7), and 0.85 gm. of dry silver oxide. The mixture was shaken with glass beads for 75 minutes, or until no free bromine compound remained in the solution. The latter was filtered and evaporated *in vacuo*. The syrup was dissolved by warming with 10 cc. of absolute methyl alcohol and the solution was allowed to cool slowly. 1.03 gm. of a crystalline product were recovered. Recrystallization from absolute methyl alcohol gave 0.84 gm. of pure β -heptaacetylgentiobiuronic acid methyl ester. The substance melted at 198–199° (uncorrected).

$$[\alpha]_D^{25} = -11.0^\circ \text{ in chloroform } (c = 1.0 \text{ per cent}); [M]_D^{25} = -7310^\circ$$

Analysis— $C_{11}H_{12}O_{10}(COCH_3)_7(COOCH_3)$

Calculated. C 48.8, H 5.46, OCH_3 4.67, $COCH_3$ 45.3

Found. " 48.4, " 5.48, " 4.64, " 46.1

Preparation of α -Heptaacetylgentiobiuronic Acid Methyl Ester—

0.5 gm. of pure β -heptaacetylgentiobiuronic acid methyl ester was dissolved in 10 cc. of acetic anhydride solution containing 1 gm. of freshly fused zinc chloride. After 14 minutes heating at 50° the

optical rotation of the solution was practically constant. The solution was poured into ice water, extracted with chloroform, and neutralized at 0° with dilute sodium hydroxide solution. The chloroform solution was evaporated *in vacuo*, and the residue was recrystallized from absolute ethyl alcohol. 0.27 gm. of material was obtained. After several recrystallizations, the derivative melted at 201–202° (uncorrected).

$$[\alpha]_D^{22} = +48.4^\circ \text{ in chloroform } (c = 0.9 \text{ per cent}); [M]_D^{22} = +32,150^\circ$$

Analysis— $C_{11}H_{12}O_{10}(COCH_3)_7(COOCH_3)$

Calculated. C 48.8, H 5.46, OCH_3 4.67, $COCH_3$ 45.3

Found. " 48.8, " 5.58, " 4.62, " 45.4

The difference in the molecular rotation of the α - and β -heptaacetates is 39,500°.

The heptaacetyl glucose glucuronide methyl ester obtained from the aldobionic acid derived from the specific polysaccharide of Type III pneumococcus melts at 250° and shows the rotation $[\alpha]_D^{22} = +41.7^\circ$ in chloroform (11). A mixture of this substance with the α -heptaacetyl methyl ester of gentiobiuronic acid shows a depression of the melting point, the mixture melting unsharply, beginning at 193°. The two aldobionic acid derivatives are therefore not identical.

SUMMARY

1. The aldobionic acid, galactose-6- β -glucuronide, has been synthesized and found to be identical with the aldobionic acid obtained from gum acacia.

2. A second aldobionic acid, gentiobiuronic acid, has likewise been synthesized and obtained in the form of its heptaacetyl methyl ester.

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A METHOD FOR THE QUANTITATIVE DETERMINATION OF PHENOLS*

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In studying the anthelmintic properties of certain alkylphenols it became necessary to determine the degree of absorption and excretion of these substances. Although satisfactory quantitative tests for many types of phenols are known and have been reviewed by Gibbs (1), an exhaustive search of the literature failed to reveal any method that was applicable to those *p*-substituted phenols which are only slightly soluble in water. Hence, it became necessary to devise a method for the estimation of these compounds.

Nitric or nitrous acid, alone or in the presence of metallic salts, has been used as a qualitative color reagent for phenols, but has had quantitative application only in the case of certain water-soluble phenols. The new colorimetric procedure described here is based upon the action of nitric acid in acetic acid solution and subsequent neutralization with alkali. Although this method was originally devised for the quantitative determination of *p*-alkylphenols, it may be employed equally as well with most other types of phenolic substances.

Method

The standard is prepared by pipetting 1 cc. of a 1:1000 glacial acetic acid solution of the phenol under investigation into a 50 cc. volumetric flask, diluting with 5 cc. of acetic acid, and then adding 6 drops of sulfuric acid and 2 drops of nitric acid. The mixture is thoroughly shaken and warmed on a steam bath until the pale

* The funds for carrying out this work were given by the International Health Division of the Rockefeller Foundation.

yellow color that develops has reached its maximum intensity, which takes approximately 2 minutes. This solution is diluted with 5 to 10 cc. of water, cooled, neutralized carefully with 15 cc. of concentrated ammonia, and made up to the mark with distilled water. On neutralization the color deepens to a clear yellow. This standard solution can be kept for several days without change in either the shade or intensity of the color.

In the quantitative determinations of phenols in biological matter, such as feces or urine, the phenol is first separated from this extraneous matter by acidifying with phosphoric acid and distilling with steam until a few drops of the distillate give a negative test for phenol. Distillates containing more than 1 part of phenol in 10,000 parts of water may be used directly for making the solution for comparison with the standard. However, very much more dilute solutions are almost invariably encountered and they must be extracted three times with ether, in order to concentrate the phenol. An aliquot of the combined ether extracts is then used.

To prepare the unknown for colorimetric comparison, an aliquot of the ether extracts, containing approximately 1 mg. of the phenol, is evaporated just to dryness on a steam bath, and the residue taken up with 5 cc. of glacial acetic acid and transferred to a 50 cc. volumetric flask. If a concentrated aqueous solution is used directly instead of its ether extract, 5 cc. are diluted with an equal volume of acetic acid.

Either of these acetic acid solutions of the phenol is then treated exactly as in preparing the standard—namely, 6 drops of sulfuric acid and 2 drops of nitric acid are added, the flask is shaken, warmed on the steam bath, diluted with 5 to 10 cc. of water, cooled, and neutralized carefully with 15 cc. of ammonia. After it has been made up to the mark with distilled water, it is compared with the standard. When the aqueous phenol solution is used, rather than the ether extract, heating must be continued from 2 to 5 minutes longer in order to bring out the color completely.

DISCUSSION

The reaction upon which this determination is based is believed to be the formation of a nitrosophenol, the alkali salt of which is

colored. Gibbs (2), in studying the action of nitric acid in the detection of phenols, found that dilute solutions act as nitrous acid with the formation of nitroso compounds. We have found that if nitrous acid was used in the method described above, a color was obtained which compared exactly with that formed by nitric acid. The reaction was, however, so very rapid that if the mixture was warmed for more than a few seconds, side reactions occurred and produced a change in the shade of color formed. On this account, the use of nitric acid is preferable. It is very unlikely that such a small amount of acid could have caused nitration, and the fact that nitric and nitrous acids gave the same colors virtually eliminates this as a possibility. In spite of the fact that there

TABLE I

Effect of the Concentration of Acetic Acid

1 mg. of *o*-phenylphenol in 100 per cent acetic acid compared with 1 mg. in varying concentrations of acetic acid. Standard set at 20.0.

Acetic acid	Time of color development	Colorimeter reading	Calculated concentration
<i>per cent</i>	<i>min.</i>	<i>mm.</i>	<i>mg.</i>
100	1	20.1	0.996
75	1	20.2	0.990
50	2 5	20.1	0.996
25	5	20.1	0.996
10	5	Reddish tint	

must be sulfuric acid present, there was no evidence of a Liebermann condensation (3) as orange or yellow colors were always obtained. If such a condensation had taken place, the indophenol produced would have imparted a green or blue color to the solution.

The success of this method depends upon the use of acetic acid as a solvent. This not only makes the method applicable to slightly soluble phenols, but it appears to be the factor responsible for the constancy of the colors obtained. Water solutions of phenols, when treated with nitric and sulfuric acids, gave varying shades and intensities of color. The importance of the strength of the acetic acid solution used may be seen in Table I. The time required to bring out the color was found to be slightly longer when the more dilute solutions were used. Although satisfactory

comparisons were obtained with 25 per cent solutions, it seems desirable to use as strong an acetic acid solution as possible.

Some phenols required a slightly longer period of heating than others in order to complete the reaction. Prolonged warming for more than 10 minutes produced side reactions that caused a change in the shade of the color ultimately obtained and consequently must be avoided. A safe rule is to heat just twice as long as is necessary to bring out the first visible sign of a color.

The amount of sulfuric or nitric acid used could be varied by as much as 100 per cent without affecting the reaction other than to change slightly the time of color development. The more reagent used, the sooner the color appeared.

TABLE II

Comparison of Known Solutions with 1 Mg. of p-Tertiary Amylphenol or o-Phenylphenol

		Colorimeter reading		Calculated amount	Error
		Standard	Unknown		
	mg.	mm.	mm.	mg.	per cent
<i>p</i> -Tertiary amylphenol	0.5	20.0	39.9	0.504	1
	1.0	20.0	20.0	1.000	0
	1.5	20.0	13.6	1.470	2
<i>o</i> -Phenylphenol	0.5	15.0	29.7	0.505	1
	0.8	20.0	24.9	0.802	0.5
	1.0	20.0	20.1	0.996	0.5
	1.2	20.0	16.1	1.242	3.5
	1.5	20.0	13.5	1.481	1
	1.8	20.0	11.4	1.754	2.5
	2.0	20.0	9.8	2.041	2

Although sodium or potassium hydroxide gave a slightly deeper color than ammonia, very concentrated solutions must be used in order to keep the total volume below 50 cc. On this account, it was found more convenient to use ammonia. A large excess of alkali was without noticeable effect.

The accuracy of this method has been found to be as great as the precision with which the individual observer can read the colorimeter. In Table II figures are given for the results obtained in the comparison, in a Klett colorimeter, of known solutions of *p*-tertiary amylphenol and *o*-phenylphenol. In no case was a greater error observed than when the same solution was used in

both cups of the colorimeter. In other experiments from 95 to 103 per cent of the *p*-tertiary amyphenol added to samples of urine was determined by the procedure described above.

This method gave a qualitative test with as little as 0.1 mg. of *p*-tertiary amyphenol, but for the most accurate colorimetric comparisons 0.5 to 1.0 mg. should be present.

TABLE III
General Application

	Compound	Color	Approximate sensitivity
			mg.
Phenolic compounds	Phenol	Yellow	0.1
	<i>o</i> -Hexylphenol	"	0.1
	<i>o</i> -Phenylphenol	"	0.1
	<i>o</i> -Nonylphenol	Cloudy	
	<i>p</i> -Hexylphenol	Yellow	0.1
	Thymol	"	0.1
	2,4,6-Dimethylphenol	Pale yellow	0.1
	α -Naphthol	Orange-yellow	0.05
	β -Naphthol	"	0.05
	Catechol	Reddish orange	0.1
	Hydroquinone	Rose	0.5
	Pyrogallol	Yellow	0.1
	Phloroglucinol	Rose	0.5
	Hexylresorcinol	Yellow	0.1
	<i>p</i> -Chlorophenol	"	0.1
	2,4,6-Trichlorophenol	Pink	0.2
	<i>p</i> -Aminophenol	Pale yellow	5
	Salicylic acid	" "	10
	<i>p</i> -Hydroxyacetophenone	" "	10
Non-phenolic compounds	Aniline	" "	10
	Toluene	" "	10
	Benzoic acid	" "	20
	Benzyl alcohol	Opalescent	10

As indicated in Table III, certain other aromatic compounds gave faint colors in high concentrations and special care should be taken to be sure they are not present in concentrations great enough to interfere with the test. It was found by running blanks on urine, feces, etc., that these substances are not present in normal biological fluids in amounts great enough to cause interference, but they might be troublesome in the analysis of other

materials, such as some pharmaceutical preparations, unless special precautions are taken.

General Application

In order to determine the extent of the application of this reaction, investigations were carried out on different types of phenolic substances. Table III lists representative examples of the types studied, together with the color produced and the minimum amount of each compound that will give a readily distinguishable color. Most simple alkylphenols or halogenated phenols, irrespective of the number or position of the substituents, gave very good colors. Some of the di- and trisubstituted compounds gave a red or reddish orange color instead of the yellow most commonly observed.

Phenols with a carbonyl group attached directly to the benzene ring, such as the salicylic acid derivatives or hydroxyphenyl ketones, gave such very faint colors, even at higher concentrations, that they cannot be determined by this method. Compounds which are themselves colored, as nitro- or nitrosophenols, are obviously unsuited for such a colorimetric determination.

It was observed that alkylphenols with side chains containing more than 7 or 8 carbon atoms gave cloudy solutions. This was due to the fact that, as the molecular weight of the alkylphenol increases, the solubility, even in alkali, decreases and those phenols which are very insoluble cannot be determined by this method. In a few border line cases, the use of aqueous or alcoholic sodium hydroxide in place of the weaker ammonia gave better results.

SUMMARY

The nitrosophenol test has been so modified by the use of acetic acid as a solvent, that it can be used for the quantitative determination for a wide variety of phenols. This is the first method to be reported that is generally applicable for the quantitative determination of *p*-substituted alkylphenols which are only slightly soluble in water.

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THE EFFECT OF GLUTATHIONE AND OTHER SUBSTANCES ON THE INACTIVATION OF CATALASES

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The protective function of catalase, which was postulated by Loew (1), has been demonstrated by Dixon (2). The latter showed that the destruction of xanthine oxidase is due to the hydrogen peroxide which is known to be formed during the catalytic reaction of this biocatalyst, and that this destruction is prevented when catalase is present.

It has been shown that the *in vitro* loss of catalase activity in crude preparations of the enzyme from certain species of marine animals and plants (3-5), requires the presence of oxygen. However, catalase preparations which are stable in the presence of oxygen can be prepared (6). It seems that, to destroy catalase, something is needed other than oxygen alone. Stern (7) in his review discusses some of the properties of anticatalase which is found in tissues, and which inhibits catalase, although never completely. The inhibition requires an oxygen or hydrogen acceptor, has an optimum pH of 6.4, and an optimum temperature of 38°, the anticatalase being rather thermolabile. Stern also discusses the properties of philocatalase, a thermolabile substance or group of substances, which may not only protect catalase from anticatalase, but regenerate the inactivated enzyme. He believed that anticatalase is probably an oxidizing enzyme. It is quite possible that enzymes, at least certain of them, have other functions in living cells besides the catalytic ones by means of which they are recognized.

Evidence is presented in this paper which indicates that the *in vitro* loss of catalase activity is largely due to glutathione and

perhaps ascorbic acid but is modified by the presence of other substances.

Methods

The details of the procedure for studying the loss of catalase activity have been given (3, 4, 8). It suffices to state that the enzyme preparations were stored and measurements were made in a constant temperature room at $20.0^{\circ} \pm 0.2^{\circ}$, the hydrogen peroxide solutions were prepared in buffer at pH 7.0 from Merck's superoxol and were approximately 1 M, the reaction time interval was 60 minutes, and the initial catalase activity of the solutions was sufficient to decompose, as a rule, 35 to 50 mg. of H_2O_2 . Clark's (9) KH_2PO_4 -NaOH mixture buffer, pH 7.0, 0.08 M, alone was used. The extracts, unless otherwise recorded, were 10 to 20 per cent and were prepared in the buffer.

Unless noted to the contrary, the pH of all the catalase solutions, as nearly as could be determined by the indicator method (and whether or not substances had been added), lay within the range 6.2 to 6.4.

Effect of Some Substances on Rate of Inactivation of Mussel Catalase—It was previously discovered that the *in vitro* loss of activity of catalases from certain marine animals and plants (3-5) is, under given conditions, a pseudounimolecular reaction. In the integrated form the unimolecular equation for the reaction is

$$k = \frac{1}{t} \ln \frac{a}{a - x}$$

where a = the initial number of mg. of H_2O_2 decomposed by 5 ml. of extract, $(a - x)$ = the number of mg. of H_2O_2 decomposed by 5 ml. of extract at any time, t , in days after the start, and k is the rate constant.

Before the effect of certain substances was investigated, it was necessary to learn whether or not dilution of the catalase solutions would change the rate of the inactivation constant k . Portions of a catalase solution were mixed with buffer and the rate constants determined. The nearly constant values of k given in Table I show that no appreciable change occurs up to half dilution. The original enzyme solution is designated as having a catalase concentration of unity. The fractional concentration is

the ratio of the volume of extract used to the total volume after dilution.

Solutions were prepared in buffer of the substances listed under Fig. 1, and carefully measured volumes were added to equal portions of crude mussel catalase preparations. k was then determined for each. Sodium disulfide caused a marked increase with increase in concentration, but sodium sulfide produced but little change. Potassium cyanide depressed the rate constant with increase in concentration. Although the catalase solutions to which sufficient amounts of this salt had been added showed no measurable loss of activity during the few days in which observa-

TABLE I
Effect of Dilution on Inactivation of Catalase

	Mussel catalase					
Fractional concentration ..	1.0	0.916	0.834	0.760	0.667	0.500
k	0.092	0.089	0.092	0.101	0.089	0.108
	Beef kidney catalase after addition of glutathione					
Fractional concentration...	1.0	0.9	0.8	0.7	0.6	0.5
n	0.149	0.146	0.149	0.209	0.146	0.232

k is the rate constant for the inactivation of mussel catalase and is given by the equation $k = (1/t) \ln (a/a - x)$; n is the inactivation constant for partially purified beef kidney catalase after the addition of glutathione and is determined by the expression $n = (1/\log_{10} t) \log_{10} (a_0/a - x)$.

tions were made, after long standing they were found to have lost their activity completely. Because of the acid reaction of ascorbic acid, an equimolar quantity of sodium hydroxide was added to a solution in buffer. This vitamin is autoxidizable but has been shown to be protected from oxidation when dissolved in extracts from animal tissues (10-12).

But few of the compounds listed in Table II caused a marked increment or decrement in the rate constant of catalase inactivation; that of the control is designated as k_0 . Golzow and Janowsky (13) avoided loss of catalase activity in diluted blood by adding ethanol (1:5000). Methanol and ethanol markedly de-

creased the rate of inactivation of mussel catalase, but glycerol and the carbohydrates had little effect. Uric acid also causes a decrease in rate, whereas potassium fluoride and thiopropionamide increase it. However, the change produced by the last may have been the result of its decomposition, with the formation of H_2S . Small differences between k and k_0 are to be discounted because of the limitations of the method. Some of the compounds, of course, were modified by other enzymes which were present.

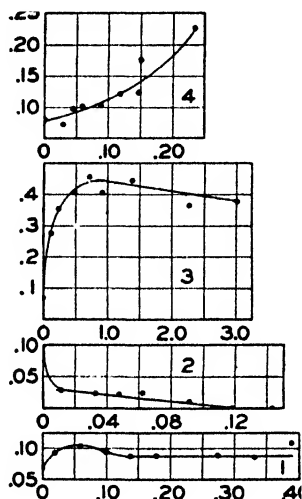


FIG. 1. Effect of certain substances on mussel (*Mytilus californianus*) catalase inactivation. The abscissæ represent mg. of added substance per ml. of catalase solution (total volume). The ordinates represent the rate constant, k , for Na_2S , Curve 1; KCN , Curve 2; neutralized ascorbic acid in terms of ascorbic acid, Curve 3; $NaHS$ in terms of H_2S , Curve 4. J. T. Baker Chemical Company c.p. $NaHS$ + aqua was used. The solution employed was specified to contain about 9 per cent available H_2S . This concentration was taken to be exact and thus the plotted points are not precise.

Effect of Certain Adsorbents—To 50 ml. portions of an extract of mussel tissue were added respectively, 2 gm. of bone charcoal, 2 gm. of powdered $CaCO_3$, 1 gm. of washed kaolin, 2 gm. of diatomaceous earth, 4 gm. of moist aged aluminum hydroxide precipitate, and 4 gm. of moist aged ferric hydroxide precipitate. Each flask was shaken intermittently during a 3 hour period and the

TABLE II

Effect of Certain Substances on Rate of Inactivation of Catalase from the Mussel, Mytilus californianus

Substance added	Concentration	<i>k</i>	<i>k</i> ₀ *	Substance added	Concentration	<i>k</i>	<i>k</i> ₀ *
	<i>M</i>				<i>M</i>		
Ethanol	0.004	0.031	0.086	<i>d</i> -Glutamic acid	0.01	0.106	0.080
	0.25	0	0.086		0.01	0.123	0.097
Methanol	0.004	0.048	0.086	<i>l</i> -Tyrosine	0.01	0.094	0.097
	0.25	0	0.086	Caffeine	0.02	0.070	0.071
<i>n</i> -Amyl alcohol	Saturated	0.143	0.143	Pyridine	0.02	0.078	0.071
				Quinoline	Saturated	0.055	0.071
Glycerol	0.23	0.088	0.086	"	"	0.196	0.143
Acetone	0.18	0.021	0.086	Nicotine	0.02	0.143	0.143
Dextrose	0.01	0.082	0.077	Hexamethylenetetramine	0.02	0.061	0.071
Levulose	0.02	0.073	0.071		0.02	0.124	0.143
<i>d</i> -Mannose	0.02	0.078	0.071				
Sucrose	0.02	0.079	0.071	Uric acid	10 + 50†	0.069	0.092
Maltose	0.02	0.054	0.071		10 + 60†	0.062	0.077
Lactose	0.02	0.075	0.071		Saturated (solid present)	0.056	0.080
Raffinose hydrate	0.01	0.143	0.143		20 + 60†	0.057	0.080
					0.1%	0.143	0.143
Glycogen	$\frac{0.02†}{n}$	0.069	0.071	Nucleic acid (from yeast)			
Inositol	0.02	0.074	0.071				
Inulin	Saturated	0.069	0.071	Potassium fluoride	<i>M</i> 0.02 (Approximate)	0.276	0.125
Asparagine	0.01	0.101	0.092		0.01	0.143	0.143
		0.093	0.097	Potassium ferricyanide			
<i>dl</i> -Alanine	0.02	0.094	0.097	Potassium ferrocyanide	0.005	0.077	0.125
<i>l</i> -Aspartic acid	0.01	0.124	0.097				
Allantoin	0.01	0.081	0.080	Thiopropionamide	0.02	0.390	0.125
Cystine	Saturated	0.091	0.089				

* *k*₀ = inactivation rate constant without added substance.

† Since the formula for glycogen is given as (C₆H₁₀O₅)_n, C₆H₁₀O₅ was taken as the molecular formula and a 0.02 *M* solution prepared. But the true concentration was 0.02/*n*.

‡ Ml. of saturated uric acid solution in buffer plus ml. of mussel extract.

solutions then filtered through Whatman No. 1 paper. The last four solutions treated had a much greater clarity after filtration than the original extract. Determinations of k were made in the solutions thus treated with possible adsorbents, and also in a por-

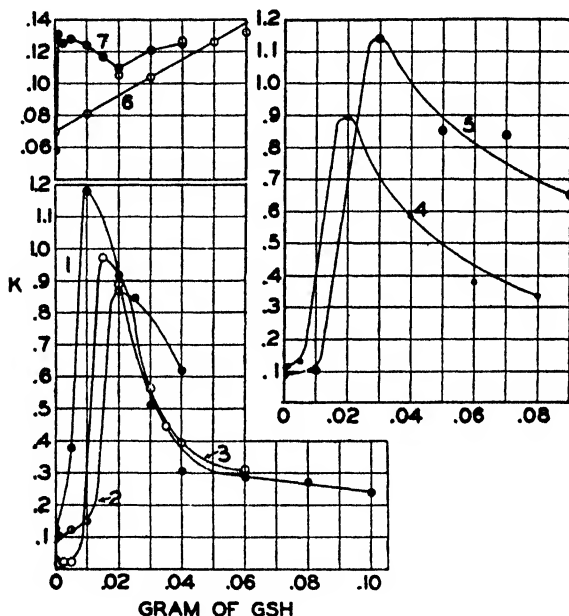


FIG. 2. Effect of reduced glutathione on the inactivation of catalases from the following: Curve 1, the clam, *Donax gouldii* (5 per cent extract); Curve 2, the sea-anemone, *Cribrina xanthogrammica* (2 per cent extract, pH 6.6); Curve 3, the California sea mussel, *Mytilus californianus*, partially purified (Morgulis, Beber, and Rabkin (6)) mussel catalase which very slowly lost its activity (8 per cent extract, pH 6.6); Curves 4 and 5, *Mytilus californianus*; Curve 6, the marine plant, *Corallina officinalis* (60 per cent extract); Curve 7, beef kidney (0.2 per cent extract, pH 6.9). The abscissæ represent gm. of reduced glutathione per 60 ml. of solution; the ordinates, the rate constant, k . Catalase from the killifish, *Fundulus parvipinnis*, yielded similar results.

tion of the original solution which had not been treated. The catalase was partly removed by all of the above except CaCO_3 , and very markedly so by diatomaceous earth, the initial activity having been reduced to 29 per cent of that of the original solution.

However, in no case did the rate constant of inactivation of the remaining catalase differ from that of the original by more than the error of the method. We infer that the following may have occurred: no adsorption of the inactivating substances took place, or these substances are catalytic or have a much higher concentration than that of the catalase, and partial removal does not affect the rate.

Effect of Glutathione on Unstable Catalase Preparations—When reduced glutathione was added in increasing quantities to equal volumes of unstable catalase solutions, remarkable effects resulted. With increase in concentration of glutathione, the rate constants for the inactivation rose sharply to a maximum and thereafter declined more slowly (Fig. 2), with the exception of catalase from the marine plant, *Corallina officinalis*.

However, it was necessary to ascertain whether or not glutathione affects the linear relationship between catalase concentration and the quantities of hydrogen peroxide decomposed under the given experimental conditions. Thus, the proportionality curves might be changed from those which consisted of a single straight line passing through the origin to those which have sharp discontinuities in slope, as was found, for example, for catalase from *Corallina officinalis* at pH 4.5 (8). If this were so, the method of deducing k would then be incorrect. However, the results plotted in Fig. 3 show that the direct proportionality still holds after the addition of glutathione. It is noted that Curve 1 is higher than Curve 2. This was due to either or both of the following factors: glutathione acts to some extent as a catalase inhibitor; a simultaneous increased rate of inactivation occurred in the 60 minute interval during which the catalase decomposed hydrogen peroxide.

Effect of Glutathione on Stable Beef Kidney Catalase—Since reduced glutathione has such a marked effect on the inactivation of unstable catalases, it was desirable to learn whether or not it would cause stable catalase preparations to lose their activity. A stable beef kidney catalase extract was prepared in distilled water, with chloroform, by the method of Morgulis, Beber, and Rabkin (6). Small volumes of this solution were added in such quantities to phosphate buffer that the activity of the resulting solutions lay within the range of the unstable catalase preparations in their

initial conditions. These solutions in buffer were colorless, considerably purer than the unstable ones, and the pH 7.0. Since c.p. chloroform contains a little alcohol as a stabilizer, the latter may have had something to do with the stabilizing of the enzyme. However, the mere addition of a small volume of chloroform to an unstable preparation does not render it stable. The quantity of active catalase present per unit weight of beef kidney is much greater than that per unit weight of mussel tissue.

When glutathione was added to these beef kidney catalase solutions and daily determinations made of the activity, it was found that the active enzyme concentration gradually decreased (Fig. 4),

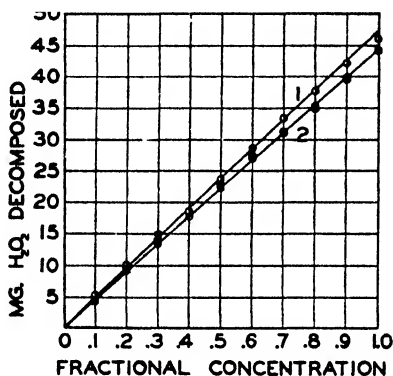


FIG. 3. Proportionality curves of mussel catalase. Curve 1, no reduced glutathione added; Curve 2, ●, ○, 2 and 4 mg., respectively, of reduced glutathione per 7 ml. (total volume including H₂O₂ solution).

but that the unimolecular equation did not satisfactorily express the processes that were occurring. When, however, the logarithm of the time, t , is plotted against the logarithm of the mg. of H₂O₂ decomposed, $(a - x)$, a more nearly straight line results, especially after the first 24 hours. For convenience, $1 + \log_{10} t$ was used (Curve 2, Fig. 4). The integrated expression for the reaction is $(a - x) = a_0 t^{-n}$ where a_0 = the mg. of H₂O₂ decomposed (proportional to the catalase concentration) by a sample at the end of the 1st day after the start, and n is the rate constant. The velocity equation is $dx/dt = na_0 t^{-n-1}$. The fact that this reaction is not of the first order may, in part at least, be ascribed to the side reaction in which glutathione passes to the oxidized form.

When small quantities of glutathione were added to 50 ml. portions of this stable preparation, n determined, and the data plotted (Fig. 4), a curve with a maximum such as was shown by the unstable preparation (Fig. 2) was not obtained. The difference in behavior may have been due to the greater purity of the stable catalase solution.

Experiments with Beef Kidney Catalase Made Unstable by Addition of Glutathione—Portions of a stable beef kidney catalase

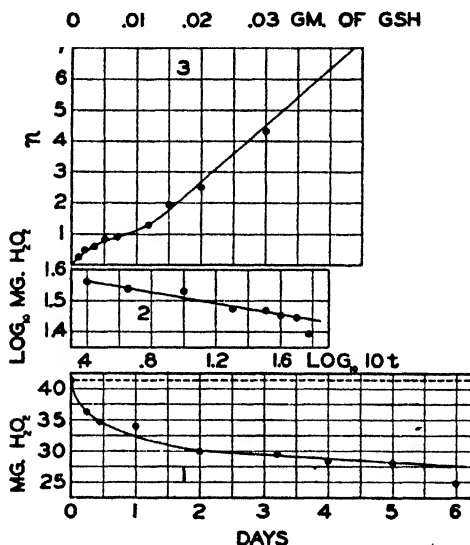


FIG. 4. Effect of reduced glutathione on stable beef kidney catalase. Curve 1, loss of activity with time after addition of 7 mg. of reduced glutathione to 50 ml. of solution; Curve 2, $\log_{10} (a - x)$, $\log_{10} 10t$ plot of the data of Curve 1; Curve 3, change in rate constant, n , with change in concentration of reduced glutathione. The abscissæ represent gm. of reduced glutathione per 50 ml. of solution.

preparation were diluted with phosphate buffer in the manner indicated in Table I, so that the total volume of each was 50 ml., 7 mg. of glutathione were dissolved in each, and the values of n determined. It is seen that the rate constants were approximately the same throughout, and thus the results compare favorably with those for unstable mussel catalase.

It was found that potassium cyanide and ethanol decrease, and sodium disulfide increases, the rate of inactivation of unstable

beef kidney catalase as well as mussel catalase. Glutathione was dissolved in a stable beef kidney catalase solution, the solution apportioned, and potassium cyanide, ethanol, and sodium disulfide added, respectively, to portions. It is seen in Table III that the results are like those obtained for unstable beef kidney catalase. Potassium cyanide was added to a quantity of the unstable,

TABLE III
Relative Effect of Certain Substances on Rate of Inactivation of Unstable Beef Kidney and Mussel Catalases and Beef Kidney Catalase Made Unstable with Glutathione

	Unstable partially purified mussel catalase				
	Original unstable solution	0.33 mg. GSH* per ml.	0.33 mg. GSH + 0.03 mg. KCN per ml.	0.33 mg. GSH per ml. under nitrogen	
<i>k</i>	0.030	0.890	0.092	Rate slower	
	Unstable beef kidney catalase				
	Original unstable solution	0.06 mg. KCN per ml.	20 mg. C ₂ H ₅ OH per ml.	NaSH added	
<i>k</i>	0.031	0.007	0	0.50	
	Stable partially purified beef kidney catalase				
	Original stable solution	0.14 mg. GSH per ml.	0.14 mg. GSH + 0.09 mg. KCN per ml.	0.14 mg. GSH + 20 mg. C ₂ H ₅ OH per ml.	0.14 mg. GSH per ml. + NaSH
<i>n</i>	0	0.10	0	Catalase activity slowly increased	0.96

* GSH = reduced glutathione.

partially purified mussel catalase solution containing 0.02 gm. of glutathione per 60 ml. (Fig. 2). Another was kept under nitrogen. The potassium cyanide nearly nullified the action of the glutathione.

The loss of activity of beef kidney catalase in unstable crude preparations was found to proceed more slowly under nitrogen than under air.

Non-Stability of Dialyzed Mussel Catalase Solutions—If glutathione and ascorbic acid are the agents which, in the presence of oxygen, cause catalases to lose their activity, it may be possible to prepare stable catalases by dialysis. A 50 per cent mussel extract in distilled water was placed in an animal membrane and dialyzed against running tap water for 24 hours; a fine stream of nitrogen was bubbled through the solution in the meanwhile. The extract was finally dialyzed for an hour against running distilled water and then filtered. However, neither this nor other dialyzed solutions of the enzyme were stable.

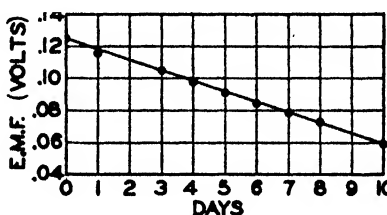


FIG. 5. Change of E.M.F. with time of a cell consisting of a normal calomel electrode and mussel extract.

Electromotive Force Studies—Since apparently an oxidation-reduction system was being investigated, it was thought that E.M.F. studies might yield some results of significance. Cells were arranged in the following way.

phosphate buffer, Pt pH 7.0, extract of mussel tissue	KCl, saturated	KCl, saturated or N;	Hg_2Cl_2 (solid)	Hg
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Temperature $20.0^\circ \pm 0.2^\circ$

Agar-salt bridges were employed, and a little toluene was poured into the mussel extracts as a germicide. Since the potential of the calomel electrode remains the same, any change in E.M.F. of the cell is to be ascribed to changes occurring in the mussel extract.

It was first found that the E.M.F. of such a cell changes linearly with time, as shown in Fig. 5. Since the potential depends upon the logarithm of the active concentration (thermodynamic activity), this curve indicates that a unimolecular reaction was occurring in the mussel extract. The substance or substances and the concentration are unknown in this instance. It remained to show

whether or not this process and the loss of catalase activity were identical. The slope ($m = d(\text{E.M.F.})/dt$) of this line is the rate constant, in volts per day, of the unidentified reaction.

If these two processes are the same, then the ratio of k/m will be constant under all given conditions. Both m and k were determined simultaneously, and the results are given in Table IV. It

TABLE IV
Change of Electromotive Force and Loss of Catalase Activity

Conditions	m	k	$\frac{k}{m}$
Under air.....	0.007*	0.112	16.0
“ “.....	0.005	0.067	13.4
Boiled extract under air.....	0	0	
Under nitrogen.....	0.005	0.038	7.6
1. Under air.....	0.060	0.045	0.75
2. “ “ portion of (1) diluted by half.....	0.060	0.045	0.75
3. $\text{C}_2\text{H}_5\text{OH}$ added to portion of (1).	0.056	0	0
4. KCN “ “ “ “ (1).	0.029	0.030	1.03

* N calomel electrode used; for all others the saturated calomel electrode was used.

TABLE V
Inactivation of Catalase from Different Groups of Organs of California Sea Mussel

Group of organs.....	Hepatopan- creas and intestines 2	Gills and palps	Mantle and gonads*	Foot† and muscles
Concentration, per cent.		8	33	15
k	0.200	0.117	0.081	0.236

* Both mantle and gonads may contain eggs or sperm.

† The foot is largely muscular tissue.

is seen that k/m is not constant, and thus the two reactions are not identical. It is to be noted, however, that the E.M.F. reaction is to be ascribed to a thermolabile substance or system, since the potential did not change with change in time for an extract portion which had previously been heated to boiling.

Rate of Inactivation of Catalase from Different Tissues—The

organs of five mussels ranging in length from 9.8 to 12.4 cm. were excised and placed in groups, as listed in Table V. Solutions were then prepared so that the initial catalase activity of each lay within 40 to 45 mg. of hydrogen peroxide decomposed in a 60 minute time interval. The rate constants were determined and are also given in Table V. It is observed that the velocity of inactivation of catalase from different groups of organs was not the same.

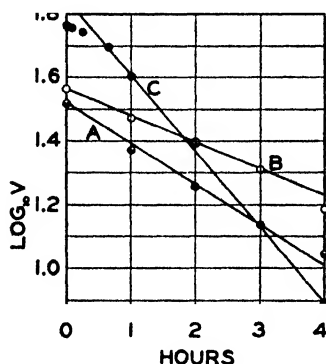


FIG. 6

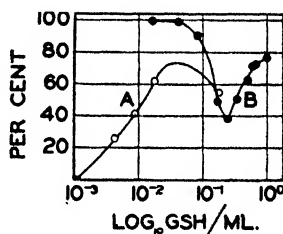


FIG. 7

FIG. 6. The oxidation of ascorbic acid; a first order reaction. Curves A and B, data of Kellie and Zilva (15); V = mg. of ascorbic acid per 100 ml. of H_2O , pH 7.4, 37° . Curve C, data of Mawson (12), ascorbic acid in "untreated" lemon juice, pH 7.5, 37° ; V = ml. of dye.

FIG. 7. Comparison of the oxidation of ascorbic acid and the inactivation of catalase in the presence of glutathione. Curve A, data of Mawson (12) for ascorbic acid; Curve B, author's data for catalase. The abscissæ represent the logarithm of the mg. of glutathione per ml.; the ordinates, per cent of control.

Measurements of relative quantities of catalase from different tissues by means of relative activities should always be made with care, since not only may the rate of loss of activity of the catalase be different for various tissues, but possible sharp changes in the slope of the proportionality curves (8) may occur. Stern (7) pointed out that, in the study of the catalase content of tissues, the influence of anticatalase has not been taken into account.

Oxidation of Ascorbic Acid—It is of interest to compare the oxidation of ascorbic acid and the inactivation of catalase which

apparently is also an oxidation reaction. Both processes require the presence of oxygen and are greatly affected by glutathione. It seems that the oxidation of ascorbic acid occurs by the introduction of two OH groups at the double bond, whether caused by the enzyme ascorbic acid oxidase (14) or otherwise. The chemical equation given by Tauber, Kleiner, and Mischkind (14) indicates that the reaction is of the first order.

Recently Mawson (12) and Kellie and Zilva (15) have studied the oxidation of ascorbic acid. In Fig. 6 are plotted semilogarithmically representative data taken from their papers. Since straight lines result, it is indicated that the reaction is unimolecular. However, the introduction of the OH groups and the required presence of oxygen show that it must be pseudounimolecular.

Glutathione has an effect on the rate of oxidation of ascorbic acid opposite to that on the rate of inactivation of catalase. In Fig. 7 are plotted some data of Mawson (12) (Curve A) for the per cent of ascorbic acid, as compared to the control, remaining after incubation for 1 hour at 37°, pH 7.4, and some data of the writer (Curve B), showing the percentage of the initial mussel catalase activity after a period of 18 hours at 20°.

These results show that if the rate constants, k , were obtained for the oxidation of ascorbic acid after the addition of definite amounts of glutathione to equal volumes of a solution of the vitamin, and in the presence of fixed concentrations of iron or copper as catalysts, the resulting curve would be the reverse of those obtained for animal catalases (Fig. 2). The curve would have a minimum rather than a maximum.

DISCUSSION

Just why methanol and ethanol have such a notable effect in decreasing the rate of inactivation of catalase, whereas *n*-amyl alcohol, glycerol, and the carbohydrates studied have little or no effect, cannot be stated.

Zeile and Hellström (16) came to the conclusion from their spectrographic data that in hemoglobin-free catalase solutions a porphyrin-iron complex is present which must be similar to hemin or an isomer. They found that both potassium cyanide and hydrogen sulfide so changed the absorption spectrum of catalase

that chemical combination was indicated. The functioning of glutathione in the inactivation of catalase is probably prevented by the combination of the cyanide with the iron of the complex and with the ions of certain heavy metals such as iron and copper which are present in solution in small concentrations.

All the animal tissues studied in this investigation gave a positive reaction for glutathione when the nitroprusside test was applied. Tissues may contain, per 100 gm., as much as several hundred mg. of extractable glutathione (17). In view of the evidence presented, we must infer that glutathione has much to do with the loss of catalase activity. Small concentrations of ascorbic acid greatly increase the rate of inactivation and may also be present in tissue extracts.

SUMMARY

1. Methanol and ethanol in extremely small concentrations (1:5000) and potassium cyanide markedly decrease the *in vitro* rate of inactivation of mussel catalase. *n*-Amyl alcohol, glycerol, and certain carbohydrates have little or no effect. Sodium disulfide, ascorbic acid, and glutathione accelerate the inactivation. The inactivation of mussel catalase in crude preparations follows a first order reaction whether or not certain given substances have been added.

2. Glutathione added to stable, partially purified beef kidney catalase renders the catalase unstable. The inactivation does not then follow the equation for a first order reaction.

3. Evidence is presented which indicates that glutathione may be an important factor in the spontaneous loss of catalase activity which occurs in mussel extracts in the presence of oxygen.

I am indebted to Professor A. Szent-Györgyi of the Institute of Medical Chemistry, Szeged, Hungary, for a supply of vitamin C, to Dr. E. M. MacKay of the Scripps Metabolic Clinic for a portion of the glutathione used, to Dr. C. E. ZoBell for the loan of certain electrical equipment, and to Dr. D. L. Fox for his kindly interest in this problem.

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PHOTOELECTRIC COLORIMETER

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In the photoelectric colorimetry of solutions, one of two factors may be the variable while the other is held constant: thickness of solution and intensity of transmitted light. The source of light is either considered constant or means are provided to compensate for its variations.

It is more difficult to use a device which depends upon the variation of solution thickness, for it is still necessary to read some electric meter to establish the relative transmission of light. Further, such arrangements do not advance visual colorimetry beyond replacing the eye with one or more photoelectric cells. The recently developed barrier-layer cell (1) has made possible an instrument which gives readings that bear a simple relationship to the concentration of solutions which conform to the fundamental Beer-Lambert law and does not require amplifying or other complex circuits which annoy the scientist as well as the technician who has little knowledge of electricity. Several variations of a simple instrument embodying these principles have been described, the most notable one being that of Müller (2). The instrument of Yoe and Crumpler (3), which also gives the transmission directly, is difficult to manipulate and requires large volumes and a delicate meter. All these instruments, however, specify optical systems which emit parallel or nearly parallel light. This specification is unnecessary and limits the instrument in two ways: first, the light-gathering lenses are limited in diameter to the width of the solution cell, resulting in low power with consequent limits upon (1) the thickness of the solution, (2) the handling of yellow solutions, and (3) ruggedness and cheapness of the meter; secondly, the volume of solution cannot be small.

The photomentering of yellow solutions is particularly difficult because their transmission in the blue end of the spectrum must be measured. The barrier-layer cell is relatively insensitive to blue light. This difficulty can be overcome by using a more concentrated beam of light.

Description and Operation of Apparatus

The instrument is shown diagrammatically in Fig. 1 and photographically in Fig. 2. *B.L.C.* represents the barrier-layer cell; *S.C.*, solution cell; *F*, light filter; *A*, microammeter, 0 to 200 microamperes, 0 to 50 microamperes; *R*₁, coarse variable shunt; *R*₂, fine variable shunt; *R*₃, fixed shunt; *S*₁, tap switch; *S*₂, toggle switch.

The solution cell is an arrangement of two cells of equal thickness on a single slide. The thickness of solution which is most useful in biochemical determinations is about 1 cm. Additional cells of 0.5 cm. and 2 cm. increase the range of the instrument. The volume of solution is from less than 1 cc. to 5 cc.

One cell is filled with the solvent, the other with the solution under test. With the solvent in position, the meter is set by means of *R*₁ and *R*₂ to read a given current. The unknown is now slid into position and the meter read again. If the reading is low, the fixed shunt is thrown out by means of the toggle switch and the reading taken from the scale with the smaller range. As will be seen under calculations, it is advisable to have a meter whose scales are calibrated logarithmically, in which case the log current is read. The ratio of current for the unknown to that for solvent is the relative transmission. The lenses have short foci and large diameters. The source of light is a 32 candle power automobile lamp which draws about 4.2 amperes from a three cell storage battery. The storage battery should be maintained at full charge. A trickle charger will answer the purpose. The lenses are each set into screens which completely divide the case of the instrument, thus preventing conduction or convection of heat. Infra-red rays and possible change in spectral output of the light source are made inconsequential by the use of a filter.

The rise in temperature was determined for a combination of yellow solution and blue filter and a blue solution with a red filter. 2 cc. of a blood creatinine standard containing 0.06 mg. of creat-

inine in 30 cc. of final volume were placed in a solution cell of 1 cm. thickness. The bulb of a thermometer was placed in the

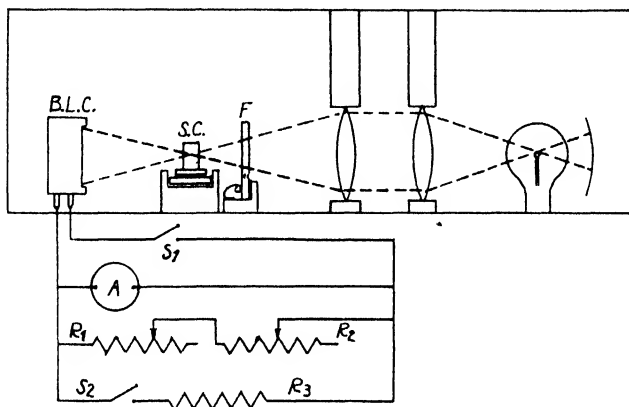


FIG. 1 Schematic drawing of the apparatus and its optical system. *B.L.C.* represents the barrier-layer cell; *S.C.*, solution cell; *F*, light filter; *A*, microammeter, 0 to 200 microamperes; *R*₁, coarse variable shunt; *R*₂, fine variable shunt; *R*₃, fixed shunt; *S*₁, tap switch; *S*₂, toggle switch.



FIG. 2. Photoelectric colorimeter

solution, away from the path of light. The light, filtered through a Corning light shade, blue-green No. 428 filter, was passed

through the solution for 10 minutes, after which the solution was stirred with the thermometer and a reading taken. The temperature rose at the rate of 0.07° per minute. Under similar conditions the rise in temperature for a blue solution of Briggs' phosphorus, containing 0.83 mg. per 100 cc. together with a Corning traffic shade, red No. 245 filter, was 0.26° per minute. The length of time required for a reading is about 1 minute.

In many methods, any appreciable change in temperature would result in very definite effects on the colorimetric reading, as, for instance, in a creatinine determination, where increase in temperature will cause a reaction by both creatine and sugar if these compounds are present in the solution.

Method of Calculation

A curve relating relative transmission and concentration is plotted on semilogarithmic paper with half a dozen solutions of known and differing concentrations. These points will lie on a straight line unless, because of ionization or other complex effects, the solution does not follow Beer's law. These effects are comparatively rare in colorimetric analysis. However, as the relative transmission in the region of absorption approaches 0, there is a deviation from Beer's law under the conditions of practical measurement. The range of analysis can then be extended by the use of a thinner solution. The use of a chart is not essential. Substituting into the line formula, $y = mx + k$, the log of relative transmission for y and concentration for x , we calculate the slope m . m is determined for several values of concentration and the average taken. Unknown concentrations are now calculated from the formula $c = (\log \text{ relative transmission} - k)/m$. If the meter is equipped with a logarithmic scale, the formula becomes simpler, $c = ((R_1/R_2) - k)/m$. k represents the value of the blank and is usually 0. R_2 , the reading for the solvent may be set at 100, the calculation then becoming $c = 0.01R_1/m$ or $c = R_1/n$, which holds for most analyses. Each combination of cell, filter, and method of analysis requires a different curve or m . Once m is determined, its use or that of a curve is equivalent to that of as many duplicate standards as were used to find m or the curve. k should be rechecked for each new batch of reagents. k may be eliminated from consideration by using the reagent blank in place of solvent in the solvent cell.

Accuracy of Instrument

The accuracy of the instrument is a function of the slope of the curve. The slope may be sharpened by (1) increasing the thickness of solution and (2) selecting a filter which transmits primarily where the solution absorbs most.

Fig. 3 shows the effect of the color of light upon the acuteness of the slope of the curve for a series of blue solutions obtained in the determination of phosphorus by the method of Briggs (4). When white or blue light is used, the curve does not pass through the origin. This is due to the yellowish blank of the reagents. For

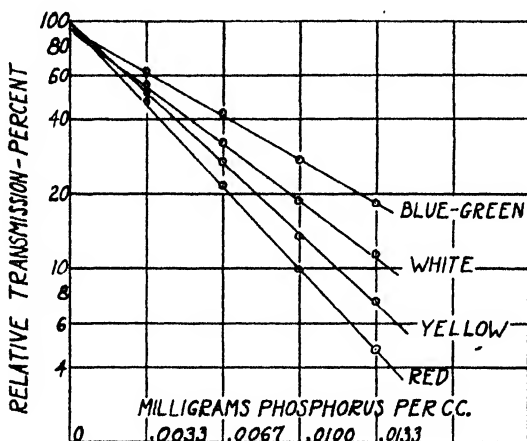


FIG. 3. Effect of the color filter

ordinary work, a selection of three or four glass filters covering as many sections of the visual spectrum is all that is necessary. The precision of the instrument is such that duplicate curves determined several months apart agreed fully within the limits of error.

Some Analyses—The method of preparing a chart is illustrated in Fig. 3. Curves for the Benedict uric acid and sugar methods are very similar to that for Briggs' phosphorus. However, for the picric acid determinations, the curves are not straight lines throughout the range of analysis, but appear as two intersecting straight lines. Likewise, the curve for nitrogen consists of two such lines. Up to a concentration of 0.2 mg. of NH_3 in 100

cc. of final solution, there is one straight line; from 0.2 mg. to about 0.6 mg., the line continues straight but at another slope.

In Fig. 4, the transmission is taken relative to the reagent blanks rather than water. The data recorded here were taken with a single range, 0 to 200 microamperes, ammeter and a solution cell of 1 cm. thickness. In preparing the known solutions for plotting the curve, it is essential in this instance, and advisable generally, to add the same quantity of reagents to each solution and that the final volumes be the same, rather than to secure standards by the

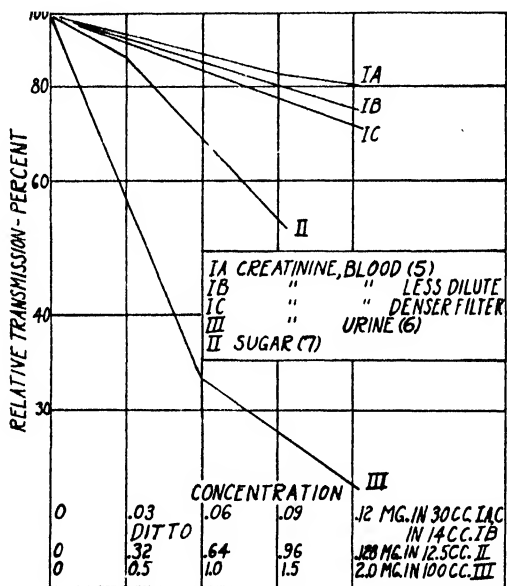


FIG. 4. Picric acid solutions

process of diluting the final solution. The Corning light shade, blue-green No. 428 filter was found satisfactory. It also serves well with the nitrogen determinations which produce a more definitely red color.

Table I shows the recovery of creatinine from a filtrate of human blood prepared in accordance with the Haden (8) modification of the Folin-Wu method. To varying amounts of filtrate were added varying quantities of creatinine standard, 1 cc. = 0.006 mg. The solutions were prepared on the basis of 20 cc. of filtrate or

20 cc. of a mixture including filtrate, standard, and water, to each of which 10 cc. of alkaline picrate are added. Table I is prepared on this basis, although the actual amounts taken were 0.35 of each ingredient.

By using a filter of narrower spectral range, such as the Corning No. 430, the accuracy may be increased.

TABLE I
Recovery of Added Creatinine from a Blood Filtrate

Filtrate	Standard added	Transmission	Total concentration as standard	Filtrate concentration as standard	Standard recovered
cc.	cc.	per cent	cc.	cc.	cc.
20	0	62.3	6.2		
10*	0	64.5	3.2		
14.3	5.7	59.5	10.0	4.5	5.5
17.2	2.8	60.7	8.3	5.4	2.9
11.4	8.6	58.0	12.3	3.6	8.7

* Plus 10 cc. of water.

TABLE II
Agreement between Duplicates of Yellow Solutions

Concentration, NH_4 per 100 cc.	Transmission	
	Duplicate 1	Duplicate 2
mg.	per cent	per cent
0.02	94	94
0.06	90.5	90
0.10	86	87
0.16	81	81
0.20	77.3	78
0.40	66.5	67.5
0.60	58	58

The blood creatinine standards gave a curve of sharper slope by a very slight modification; instead of diluting the standard to 20 cc. as called for in the method, dilute to 4 cc. and add the usual 10 cc. of alkaline picrate reagent. This is not objectionable in view of the small quantity of solution we require. In applying this modification to blood filtrate, 4 cc. of the usual filtrate may be taken.

Nitrogen was determined by the method of Folin and Bell (9). Mr. Myles Braver, of this laboratory, made the determinations presented in Table II in the course of his regular work. They are presented to show the extent of agreement between duplicates. The duplicate readings were taken on different days. A 1 cm. solution cell and a Corning filter No. 428 were used.

The time required for taking the reading, rinsing the cell, and refilling it for the next determination is about 1 minute. A convenient device for emptying the cell is a rubber bulb with a glass tube which is drawn to a fine bore.

SUMMARY

A photoelectric colorimeter is presented which (1) has wide range, (2) is accurate at high and low concentrations, (3) requires less than 5 cc. of solution, (4) is easily and rapidly manipulated, (5) gives satisfactory results with a rugged and inexpensive meter even when used with yellow solutions, and (6) its readings bear a simple relationship to the concentration of solutions which conform to Beer's law. A fixed shunt extends the scale of the meter.

The writer wishes to express his gratitude to Dr. K. G. Falk and Miss Grace McGuire for their valuable advice and encouragement. He is also indebted to Professor Ralph Müller of New York University for discussing with him the problems involved. This work was made possible by a Works Progress Administration project sponsored by and under the supervision of the Bureau of Laboratories of the Department of Health, City of New York.

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THE ATTEMPTED USE OF CRYSTALS AS CALCIUM ELECTRODES *

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Tendeloo (1) recently reported the successful use of fluorite crystals as electrodes for the potentiometric determination of calcium concentrations in calcium nitrate solutions, with and without gelatin, and in milk. He measured the electromotive force of the cell

Ag | AgCl | (Solution 1) CaCl_2 | CaF_2 | (Solution 2) $\text{Ca}(\text{NO}_3)_2$ | KCl (saturated) | KCl (0.1 M), Hg_2Cl_2 | Hg

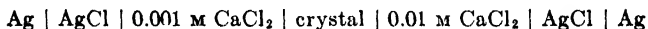
Some earlier unpublished results will be given below which show that Tendeloo has not proved that his crystal behaved as a calcium electrode. It is not intended to imply that such electrodes are impossible.

5 years ago the attempt was made to measure the electromotive force of what was essentially this same cell with fluorite or calcite crystals but with calcium chloride in both Solutions 1 and 2. The results were of two types. Either the resistance of the cell was too high to obtain measurements even with a Compton electrometer or the readings were most easily explained as including liquid junction potentials probably arising in some minute crack in the crystal. The crystals were 0.01 or 0.02 cm. thick and 0.1 to 1.0 sq. cm. in area. Since, in those experiments in which E.M.F. measurements failed, the currents detectable by the electrometer were of the order of 10^{-14} or 10^{-15} amperes, the crystal resistances were extremely high. This is in agreement with the published values (2) for the specific resistances which, measured in air, are from 10^{14} to 10^{22} ohms for calcite and 10^{17} ohms for fluorite.

* This work was done while the author was an instructor in the Department of Physiology at Yale University.

Where measurements were obtained, the differences in the E.M.F. of the cell with varying concentrations of Solution 2 were, with one exception, of the same sign and usually were of approximately the same size as when liquid junctions were included between the same solutions. This was shown in a few cases by making a pinhole through the paraffin seal around the crystal. If the crystal had had the properties of a calcium amalgam, as would be expected theoretically if no leaks were present, the differences with varying concentrations of calcium chloride would have been somewhat larger and of the opposite sign. Moreover, the differences in the E.M.F. between various concentrations of calcium chloride solutions were not specific for calcium, since they were abolished by adding equal, high concentrations of sodium chloride to each solution.

The true concentration cell



was also measured. With calcium amalgam replacing the crystal, the E.M.F. for this cell is greater than 0.077 volt ((2) pp. 326-327), while for the same cell with a liquid junction, it is about 0.036 volt. In two experiments with crystals with which measurements could be made, the values were 0.034 and 0.035 volt. It was evident that here also the crystal allowed liquid contact through it.

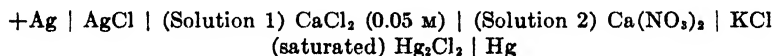
On the basis of these results no final conclusion could be reached regarding the question of using such crystals as specific electrodes for calcium. A search of the literature showed that the E.M.F. of cells containing solutions in contact with solid electrolytes had been measured by Haber and Beutner (3). As expected, they found that they could replace the metal by the solid salt in some cases. This gave support to the idea, although the resistances of the salts which they used were relatively low, and justified further efforts to make measurements across calcite or fluorite by using much thinner, but still intact crystals, or by increasing their conductivity. However, it has not been possible to continue that work up to the present time.

Haber and Beutner also studied the E.M.F. of cells containing a pellet of calcium hydroxide and calomel soaked in a mixture of vaseline and paraffin. The same idea was tried with calcium oxalate, since it is much less soluble in water than calcium hydroxide. The pellets were made from calcium oxalate alone,

a pressure of from 50 to 100 kilos per sq. cm. being used, and were then soaked in hot paraffin for varying lengths of time. After cooling, the paraffin was scraped from the surfaces and they were then used in the same way as the crystals. In some cases the resistances were again too high to measure and in others there was the same evidence of liquid junction potentials. However, some pellets gave differences of E.M.F., when the concentration of the calcium chloride solution was changed, that were of the correct sign for a calcium electrode. Unfortunately the values obtained were too erratic to be useful quantitatively.

When Tendeloo's results were examined, no crucial evidence was found that the crystals had behaved as electrodes, specific for calcium. In response to a direct inquiry Tendeloo stated that "high sodium chloride concentrations caused trouble." Although the polarity of his cell was not mentioned, the mercury electrode was positive in the outside circuit unless the crystal itself had an E.M.F. due to asymmetry. With that polarity, if calcium nitrate behaved like calcium chloride, as it should because of its similar transference number, the sign and magnitude of his differences in E.M.F. at various salt concentrations were those to be expected if also they arose from liquid junctions.

A few additional experiments were performed to check this. The cell



was measured. A paraffined paper replaced the fluorite crystal and the liquid junction was made in a pinhole through the paraffin. Since Tendeloo used a 0.1 M calomel cell, the data have been changed by 0.092 volt. This changed the sign of the mercury electrode from negative to positive. The E.M.F. of the cell with 0.5 M calcium nitrate as Solution 2 minus the E.M.F. with 0.05 M calcium nitrate as Solution 2 was 0.015 volt, compared with Tendeloo's value of 0.014 volt. Similarly the difference between 0.05 M and 0.005 M calcium nitrate was 0.014 to 0.019 volt, compared with Tendeloo's value of 0.0135 volt. In this case the results were erratic and there was undoubtedly a flow from one solution into the other.

The data of Tendeloo on the use of the crystals in calcium nitrate solutions containing gelatin gave no additional evidence regarding

the source of the differences in E.M.F. However, two points in connection with his measurements in milk were of interest. First, a reasonable value for calcium concentration was obtained. E.M.F. measurements with the perforated paraffin disk in milk diluted with an equal volume of water, when used as though the milk was a solution of pure calcium nitrate, gave a value of about 0.005 M calcium compared to Tendeloo's value of 0.008 M. As the liquid junction potential depended on the mixture of salts in the milk, the value for the calcium concentration calculated from it had no meaning.

The second point was the fact that Tendeloo observed no change in E.M.F. until 0.018 M calcium nitrate had been added to the milk. This was explained as due to the complete binding of smaller amounts of calcium. If a liquid junction was the site of the change in E.M.F., the change with small amounts of added calcium nitrate would be much less than in pure calcium nitrate solutions because of the other salts present in the milk.

In other words the data of Tendeloo, as presented, can be interpreted more readily on the assumption that his cells contained a liquid junction at the crystal rather than an electrode, specific for calcium. If the measured differences of E.M.F. were due to different liquid junctions, the results in gelatin solutions and in milk also require reinterpretation.

It perhaps should be repeated that the purpose of this report is not to imply that a true calcium electrode of the type described cannot be obtained, but to indicate the difficulties involved before the question can be finally tested experimentally.

SUMMARY

Some experiments on the attempted use of calcite and fluorite crystals as calcium electrodes, which failed because of the high electrical resistance, have been described. A recent paper reporting success in similar experiments has been criticized and the conclusion reached that the experimental feasibility of this type of calcium electrode is still an open question.

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THE LIPOID PHOSPHORUS CONTENT OF HYPERTROPHIED HEARTS AND KIDNEYS IN THE RAT*

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Indirect evidence for a relationship between physiological activity and phospholipid composition of a tissue has been presented chiefly by Bloor and his associates (1). On the basis of studies of the phospholipid content of such tissues as the corpus luteum (2), mammary gland (3), tumors (4), Wharton's jelly (5), and muscle (3, 6), the hypothesis has been formulated that the percentage concentration of phospholipid varies directly as the activity of the tissue.

If Bloor's hypothesis is correct, the phospholipid concentration of the hypertrophied heart and kidney should be increased. Cardiac hypertrophy can be produced secondarily to hypertension in partially nephrectomized rats (7); and protein ingestion affects the degree of renal hypertrophy in the intact and unilaterally nephrectomized rats (8). These experimental conditions afford a direct method for testing the relationship between physiological activity and phospholipid content of the heart and kidney. The results obtained in these experiments are not in agreement with Bloor's hypothesis.

Methods

Albino rats of Wistar strain were used in these experiments and were maintained on a stock diet until they were between 60 and 70 days old. At this time they were subjected to operation. The operative procedure has been reported elsewhere (7). The intact rats were subjected to the procedure of exposure and replacement

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of the two kidneys; the unilaterally nephrectomized rats had the left kidney exposed and replaced and after a week the right kidney was removed. Subtotal nephrectomy was performed by a two stage operation; about two-thirds of the left kidney was cut off from its circulation by ligation of the two poles; a week later the right kidney was excised. In addition, a group of animals was subjected to these respective procedures at this time and was sacrificed immediately for analysis of kidneys. These animals are designated as stock controls.

Immediately after the operation the animals were placed on one of the experimental diets listed in Table I. These diets differed only in the percentage concentration of the extracted meat which

TABLE I
Composition of Experimental Diets

Diet No	E.M. 10	E.M. 20	E.M. 40	E.M. 60	Stock diet (Bal Ra)*
Extracted meat*	10	20	40	60	
Starch.....	60	52	32	12	
Lard.....	16	14	14	14	
Cod liver oil.....	5	5	5	5	
Salt mixture†	4	4	4	4	
Yeast.....	5	5	5	5	
N content, <i>per cent</i>	1.87	3.18	5.90	8.49	5.63

* Obtained from Valentine's Meat Juice Company, Richmond, Virginia.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919).

was the principal source of protein. To prepare the extracted meat lean beef muscle was ground and thoroughly extracted with hot water, pressed free of fluid, dried in a steam-heated container, and ground to a fine powder. A maximum of eight rats was allowed to a double bottom cage, and food and water were always available. When the animals were sacrificed at variable intervals after operation, they were all in good health and more than 4 months old.

After the animals were sacrificed, the hearts and kidneys were immediately removed. The heart ventricles were separated from the atria, blotted free of blood, and weighed. The kidney was dissected free of fat, dried on filter paper, and weighed. These

tissues were immediately macerated in a mortar with the aid of ground glass. The extraction of the lipids was done according to the method of Osato and Heki (9). The lipid phosphorus was determined in this extract according to the procedure recommended by Man and Peters (10).

TABLE II

Lipoid Phosphorus Content of Non-Hypertrophied and Hypertrophied Hearts

Group	No. of animals	Diet	Lipoid P Heart weight $\times 100$	Lipoid P $\times 100$ Surface area	Heart weight $\times 100$ Surface area
			mg.	mg.	mg.
I. Intact	10	E.M. 10	120 \pm 7.40*	0.20	168
	8	" 20	124 \pm 7.05	0.21	168
	13	" 40	117 \pm 7.95	0.20	167
	10	" 60	117 \pm 7.55	0.20	170
II. Unilaterally nephrectomized	8	" 10	125 \pm 7.40	0.22	172
	6	" 20	123 \pm 7.05	0.20	160
	9	" 40	121 \pm 7.95	0.21	163
	6	" 60	116 \pm 7.55	0.20	169
III. Partially nephrectomized animals with heart weight to surface area ratios within normal limits of Groups I and II	10	" 10	120 \pm 6.85	0.22	181
	9	" 20	120 \pm 5.85	0.21	165
	10	" 40	120 \pm 9.25	0.20	166
	12	" 60	122 \pm 7.95	0.21	167
IV. Partially nephrectomized animals with heart weight to surface area ratios above normal limits of Groups I and II	9	" 10	115 \pm 5.00	0.26	231
	16	" 20	122 \pm 8.25	0.26	213
	14	" 40	120 \pm 7.95	0.25	205
	5	" 60	118 \pm 12.00	0.25	207

*The mean deviations were obtained from both the intact and the unilaterally nephrectomized groups.

The degree of hypertrophy of the heart and kidney was expressed by the formula (heart or kidney weight/surface area) $\times 100$, representing the mg. of heart or kidney tissue per 100 sq. cm. of surface area. Similarly, (total lipid phosphorus/surface area) $\times 100$ was calculated to express the concentration in mg. of lipid phosphorus per 100 sq. cm. of surface area. The surface area

was calculated by the formula of Lee (11) from the weight of the animal at the time of sacrifice. The mean deviation of the mean was calculated according to the formula recommended by Scott (12).

Results

Heart—Table II presents the data showing the lipid phosphorus content of the hearts of intact, unilaterally, and partially nephrec-

TABLE III

Lipoid Phosphorus Content of Kidneys in Intact and Unilaterally Nephrectomized Rats Fed on Different Diets

Group	No. of animals	Diet	$\frac{\text{Lipoid P}}{\text{Kidney weight}} \times 100$	$\frac{\text{Lipoid P}}{\text{Surface area}} \times 100$	Value in per cent of stock control	$\frac{\text{Kidney weight}}{\text{Surface area}} \times 100$	Value in per cent of stock control
			mg.	mg.		mg.	
Intact	12	E.M. 10	134 \pm 9.05*	0.55	75	406	80
	8	" 20	141 \pm 8.60	0.61	84	429	84
	14	" 40	137 \pm 9.75	0.68	93	492	96
	12	" 60	141 \pm 11.40	0.76	104	534	105
	12	Stock control	142 \pm 6.40	0.73	100	510	100
Unilaterally nephrec- tomized	8	E.M. 10	140 \pm 9.05	0.46	132	324	131
	7	" 20	141 \pm 8.60	0.44	126	308	125
	8	" 40	137 \pm 9.75	0.53	152	376	152
	6	" 60	132 \pm 11.40	0.60	172	460	186
	12	Stock control	142 \pm 6.40	0.35	100	247	100

* The mean deviations were obtained from both groups.

tomized rats on the various diets. Cardiac hypertrophy was not considered definite unless the heart weight to surface area ratio exceeded the highest value obtained in the same control dietary groups. It is seen that the percentage concentration of lipid phosphorus is constant in all groups. The lipid phosphorus per unit of surface area is also constant in all animals with heart weight to surface area ratios within the normal range, but is elevated in those animals with hypertension and cardiac hypertrophy.

Kidney—Table III presents the data showing the lipid phosphorus content of the kidneys of the intact and unilaterally nephrectomized rats on the various diets. The effect of diet on the degree of kidney hypertrophy was made by a comparison with the respective stock controls.

The percentage concentrations of lipid phosphorus are relatively constant in the various groups. It is noted that the kidneys in general are larger per unit of surface area as the percentage of protein in the diet increases. With the stock controls as a standard of 100 per cent, it is seen that the percentage values for kidney hypertrophy and phospholipid concentration per unit of surface area closely parallel each other in the intact and unilaterally nephrectomized groups.

DISCUSSION

Bloor (3) stated that extra work in a tissue results in normal hypertrophy with an accompanying increased percentage of phospholipid. This idea was based on a comparison of different skeletal muscles of variable activity in the same animal and of the same muscle in different species of the same animal. There is no evidence, however, to show the effect of hypertrophy in the same muscle or organ in animals maintained under the same conditions. In the present experiments, hypertrophy of the heart was a result of hypertension. A relationship between the degree of cardiac hypertrophy and the height of blood pressure has been demonstrated (13). Kidney hypertrophy was produced by feeding diets varying in protein content.

The phospholipid concentration of the hypertrophied heart was not increased above that of the non-hypertrophied heart. A similar result was obtained for the creatine concentration of these hearts (14). Since these experiments were performed on rats of the same strain, the various groups being maintained under the same dietary and experimental conditions, it is felt that the negative results obtained for the lipid phosphorus are significant. It was likewise noted that the phospholipid concentration of the hypertrophied kidneys in both the intact and unilaterally nephrectomized rat was not affected.

It has been shown that the amount of phospholipid per unit of surface area in both the heart and kidney parallels the degree of

hypertrophy without any accompanying percentage increase in concentration. Since the function and activity of these two tissues are so different, the evidence appears to indicate that phospholipids form an integral part of the cell protoplasm and are increased proportionately to cell hypertrophy.

SUMMARY

The hearts and kidneys of intact, unilaterally, and partially nephrectomized rats fed diets containing 10, 20, 40, and 60 per cent extracted meat were analyzed for lipid phosphorus.

There is no increase in the percentage concentration of lipid phosphorus in the hypertrophied heart and kidney. The lipid phosphorus content per unit of surface area parallels the degree of hypertrophy in the heart and kidney.

The data presented do not favor Bloor's hypothesis regarding the relationship between phospholipids and physiological activity.

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THE DETERMINATION OF CREATININE WITH SODIUM 3,5-DINITROBENZOATE

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The reaction of picric acid with creatinine in alkaline solution (Jaffe's reaction), which is the basis of the Folin method (1) for the determination of creatinine, has been thought to be either a reduction of the acid or else the formation of a red tautomer of creatinine picrate. Greenwald (2) obtained evidence that the tautomerism involves picric acid as well as creatinine, and that other nitro compounds give no color under the conditions used (2-4). The reaction, therefore, has been thought to be peculiar to this one acid. However, similar rearrangements probably account for the color in alkaline solution of many nitro compounds, such, for instance, as trinitrobenzoic acid, which dissolves in sodium carbonate to give a pale yellow solution of the sodium salt, but gives a deep red color with excess of sodium hydroxide. Any nitro compound which behaves in this way toward alkali is obviously unsuitable for use in place of picric acid in the Folin procedure.

It has been found in this laboratory that 3,5-dinitrobenzoic acid gives a brilliant garnet-red with creatinine in alkaline solution and can be used for its determination. Under similar conditions, the acid alone gives no red. We have repeatedly purified the acid by recrystallization from alcohol and obtained a product which did not change in its color-producing qualities as purification proceeded. The chromogenic property reached a maximum with the maximum purity. On the other hand, one batch of the crude acid, as purchased, contained a yellow substance which influenced the reaction in such a way that the color obtained was very pale and very quickly destroyed. Greenwald and Gross (4), who have

overlooked the color which is given by the acid, may have done so because of the presence of this impurity in their solutions.

The behavior of the new reagent toward creatinine of urine and of blood has been compared with that of picrate, and its use has been found to be advantageous. When pure, the reagent gives a very pale yellow solution of the sodium salt, which color is negligible when amounts of creatinine greater than 0.025 mg. are determined. With amounts greater than this, the color is easily read in a colorimeter and can be attributed entirely to the creatinine without detectable error. Furthermore, the reagent develops less extraneous color than picrate with certain substances such as glucose and acetoacetic acid, which frequently occur in urine and blood. Consequently, the values obtained by analysis of such solutions are more trustworthy than those obtained with the Folin procedure.

EXPERIMENTAL

Purification of 3,5-Dinitrobenzoic Acid

The acid, purchased from the Eastman Kodak Company, melted from 200–204°. Of this impure acid, 50 gm. were dissolved in 100 cc. of boiling 80 per cent alcohol. The solution was filtered and cooled to about 5°, whereupon crystals formed within a few minutes. After about $\frac{1}{2}$ hour at 5°, the crystals were filtered off and were washed with 50 per cent alcohol. For use, in this instance, a second recrystallization was necessary in order to remove the yellow impurity more completely. The purified acid was very pale yellow in color and melted at 204–204.5° (capillary tube).

Preparation of Reagent (6 Per Cent Sodium Dinitrobenzoate)

The acid is not readily soluble in water, but dissolves in sodium carbonate solution with evolution of carbon dioxide. 30 gm. of the acid were suspended in 420 cc. of water, and 80 cc. of 10 per cent sodium carbonate were added. When no more of the acid dissolved, the solution was filtered and was ready for use.

The reagent was nearly colorless, but several preparations were comparable in color to a solution containing 1 cc. of saturated picric acid (room temperature) in 5 liters of water. A large part of the acid may be recovered by adding HCl to the solution after the determination. Recrystallization from alcohol in the presence of

norit to remove adsorbed colored material yields a product suitable for further use.

Determination of Creatinine in Urine

Reagents—

6 per cent sodium 3,5-dinitrobenzoate.

5 per cent sodium hydroxide.

Standard solution of creatinine in 0.1 N HCl.

*Procedure—*To 1 cc. of creatinine standard (\approx 1 mg. of creatinine in 0.1 N HCl) in a tube graduated at 25 cc., and to 1 cc. of urine in another, are added 20 cc. of 6 per cent sodium dinitrobenzoate (graduated cylinder)¹ and 2 cc. of 5 per cent sodium hydroxide. The solutions, diluted at once to 25 cc., are mixed, and, after 10 minutes, comparisons are made in a colorimeter. The calculation is as usual. The reactions with the standard and the unknown must be carried on within 5 minutes of each other and the comparison should be made within 30 minutes after completion of the color development. If solutions with less color are desired, dilution may be made to 50 cc. without decrease of stability of color. If diluted to 100 cc., 5 per cent fading occurs in 5 minutes.

*Observations on Color—*The reaction of 3,5-dinitrobenzoate with creatinine corresponds to that of picrate in that a large excess of the reagent must be used for full color development. With the amount stated, the color develops rapidly (10 minutes) from violet to garnet, which remains for 5 minutes, and then fades slowly (1 hour) through crimson to reddish brown. The hue and intensity of the color depend upon the purity of the 3,5-dinitrobenzoate, the color with impure reagent being definitely inferior. With less than 20 cc. of 6 per cent dinitrobenzoate, complete color is not evolved and the changes in intensity and hue are more rapid. With 2 cc. of 5 per cent NaOH, a pink color (indicator effect) may be formed with some preparations of dinitrobenzoate in the absence of creatinine, although with the pure reagent such color is

¹ 15 cc. of 3 per cent sodium dinitrobenzoate and 2 cc. of 5 per cent sodium hydroxide may be used, in which case the color is less deep by about 25 per cent and is less stable. It is desirable that the standard and the unknown be made simultaneously and that the reagent be measured with a pipette.

inappreciable. This indicator color may be removed by the careful addition of KH_2PO_4 without destroying the color due to creatinine. A 0.2 M solution of KH_2PO_4 (27.2 gm. per liter) is convenient, and the amount to be added is determined readily with a control tube.

Fading is more rapid than with picrate and depends to a large extent upon the alkalinity of the solution. The color is destroyed at once by acid and returns when the solution is made alkaline. With less alkali than is prescribed, the color is not proportional to the creatinine content; with more alkali, the reaction is too rapid. Under the conditions given (2 cc. of 5 per cent NaOH and 20 cc. of 6 per cent dinitrobenzoate) there is an interval of about 5 minutes after the maximum color development when fading is not noticeable. This constancy of color may be attributed to equal rates of color development and of fading.

TABLE I
Determination of Creatinine in Water Solution

The values are given in mg. per cc.

Present.	0.60	0.80	1.20	1.40	1.60
Found.	0.60	0.83	1.22	1.42	1.60

Each solution was compared against the same standard which contained 1.0 mg. of creatinine.

The sensitivity of the reagent was tested toward a number of substances, several of which are known to interfere with the color in the picrate procedure. No color is developed with glucose in 10 per cent solution, nor with creatine, arginine, methylguanidine, *as*-dimethylguanidine, guanidine, fructose, and cystine. Less color is given than by picrate with acetone and acetoacetic acid, but the reagent is similarly sensitive toward uric acid, furfural, and formaldehyde. Amounts of uric acid 10 times greater than are present in normal urine do not interfere in the determination of creatinine.

That the color obtained is proportional to the amount of creatinine is illustrated by the data of Table I, in which water solutions of creatinine were analyzed.

In order to determine whether or not the values for urine are the

same as those given by picrate, determinations with the two reagents were made. Purified picric acid was used throughout. The values obtained with picrate are slightly lower than those with dinitrobenzoate, but the differences are not significant. The figures given (Table II) are typical of a number of analyses which were made.

TABLE II

Comparison of Picric Acid and 3,5-Dinitrobenzoic Acid for Analysis of Urine for Creatinine

The values are given in mg. per cc.

Urine No.	I	II	III	IV	V	VI
Picric acid	2.54	1.72	1.21	0.84	1.57	0.99
Dinitro "	2.58	1.83	1.24	0.85	1.62	1.02

Urine I represents urine collected after ingestion of creatinine; Urine II, 10 cc. of Urine I + 5 cc. of water; Urine III, 5 cc. of Urine I + 5 cc. of water + 5 cc. of creatinine solution (\approx 5 mg. of creatinine); Urines IV, V, and VI were normal urines. Urine I was modified as indicated in order to demonstrate that no substance other than creatinine of the urine influenced the color.

Determination of "Apparent Creatinine" of Blood

Reagents—

Saturated solution of sodium 3,5-dinitrobenzoate, made from 20 gm. of dinitrobenzoic acid, 150 cc. of water, and 50 cc. of 10 per cent sodium carbonate.

10 per cent sodium hydroxide.

Standard solution of creatinine in 0.1 N HCl.

Procedure—To 10 cc. of tungstic acid filtrate (Folin-Wu) in a test-tube and to 0.01 or 0.005 mg. of creatinine in 10 cc. of solution in another are added 3 cc. portions of saturated sodium dinitrobenzoate and 0.5 cc. of 10 per cent sodium hydroxide. The tubes are shaken and allowed to stand for 10 minutes. Within the following 10 minute interval the solutions are compared in a colorimeter. The readings are referred to a graph (see below) from which the creatinine content of the filtrate is established.

Construction of Graph of Creatinine Values—When determinations of creatinine are made upon amounts smaller than 0.03 mg. per 10 cc. of solution, the color obtained is not proportional to the

creatinine content. A similar statement is true for the picric acid procedure (5). Before accurate values are obtained it is necessary that solutions containing from 0.003 to 0.03 mg. portions of creatinine per 10 cc. be carried through the above reaction and compared one with another. Graphs correlating the colorimeter readings with creatinine concentrations can then be made. These graphs show that high values for creatinine of blood filtrates result when calculations are made in the usual way.

Observations Regarding Color—Because of the small amount of creatinine in filtrates from normal blood, concentrated reagents are necessary to give sufficient color for use in a colorimeter. The 3 cc. of saturated dinitrobenzoate are most convenient, but deeper colors may be obtained by dissolving 0.3 gm. of dry sodium dinitrobenzoate in the 10 cc. of filtrate. Fading is rapid, and the time interval between completion of the color development and comparison must be correspondingly short. Conditions for greater stability of color were sought for without success.

Comparison of Picrate and Dinitrobenzoate Procedures for Creatinine of Blood

Whereas the picrate values for creatinine of retention bloods usually are acceptable, those reported in bloods with normal or low creatinine content are subject to serious doubt (5-7). The color due to the picrate alone is enormously deep as compared with that due to the creatinine, and there seem to be substances in blood other than creatinine, which react with the picrate to produce extraneous color. The first of these objections does not apply to determinations with dinitrobenzoate, inasmuch as the color obtained with a filtrate is much deeper than with the reagents alone. The second objection applies much less to the dinitrobenzoate than to the picrate procedure, as the reagent is more specific in its reaction, and as the interfering brown color from the filtrate can be judged with more accuracy.

For comparison of the two procedures (8), tungstic acid filtrates were used. Each filtrate represented mixed blood from three or more patients, all with normal or slightly high urea nitrogen. They were analyzed by both procedures; the figures are recorded in Table III. The picrate values, except for two, are higher by about 0.4 to 1.0 mg.

In order to establish the more accurate values, filtrates were analyzed by both procedures, and then the creatinine of similar portions of filtrate was removed by adsorption upon Lloyd's reagent and determined after liberation from the adsorbent by use of MgO, as described by Gaebler (5). The analyses are recorded in Table IV.

The values for adsorbed creatinine, as determined by the two reagents, agreed within the experimental error of the methods and were essentially the same as those obtained by use of dinitro-

TABLE III
Picrate and 3,5-Dinitrobenzoate Analyses of Blood for Creatinine

Filtrate No.	Picrate	Dinitro- benzoate	Filtrate No.	Picrate	Dinitro- benzoate
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	1.2	0.6	11	1.0	0.38
2	1.0	0.5	12	0.6	0.1*
3	1.0	1.0	13	2.1	1.6†
4	0.5	0.4	14	1.1	0.1*
5	0.9	0.5	15	1.03	0.70
6	1.2	0.6	16	0.95	0.30
7	1.0	0.5	17	1.08	0.50
8	1.0	0.44	18	1.48	1.0
9	0.98	0.32	19	1.54	0.9
10	1.2	0.8	20	1.1	0.35

Values for both procedures were determined from graphs. Bloods had been drawn 4 or 5 hours before analysis.

* Filtrates 12 and 14 were matched against a blank; the creatinine color was doubtful.

† A blood with high creatinine content is included in Filtrate 13.

benzoate without adsorption of the creatinine. It seems, therefore, that with dinitrobenzoate, very nearly true values for creatinine of blood may be obtained directly upon filtrates. This cannot be said of the picrate procedure.

Creatinine 3,5-dinitrobenzoate may be prepared by mixing hot alcohol solutions of creatinine and of 3,5-dinitrobenzoic acid. The salt separates almost immediately, and may be filtered off with nearly quantitative yield when the solution has cooled. After recrystallization from hot water (80 cc. per gm.) or from 50 per

cent alcohol (1.6 gm. per 100 cc.) it is white, crystalline, and anhydrous. It decomposes with effervescence at a temperature which seems to depend on the rate of heating, usually between 230–240°. The creatinine content, determined colorimetrically, is 35 per cent. The calculated value is 34.7 per cent. The salt is not readily soluble in water (about 0.035 gm. in 100 cc. at 20°), but dissolves when sodium bicarbonate is added. Owing to the instability of creatinine in alkaline solution, the salt is not recommended as a standard for creatinine determinations.

TABLE IV

Creatinine of Filtrates before and after Adsorption upon Lloyd's Reagent
The values are given in mg. per 100 cc.

Filtrate No.	Without adsorption		Adsorption	
	Dinitrobenzoate	Picrate	Dinitrobenzoate	Picrate
1	0.6	1.0	0.5	0.6
2	0.3	0.9	0.4	0.5
3	0.3	0.9	0.4	0.6
4	0.45	1.1	0.50	0.70
5	0.7	1.2	0.47	0.66
6	0.65	1.3	0.50	0.64
7	0.52	0.96	0.60	0.57
8	0.52	1.2	0.65	0.60
9	0.42	1.1	0.65	0.56
10	0.5	0.9	0.7	0.6

Creatinine in amounts up to 0.3 mg. per 10 cc. in pure solution was accounted for quantitatively by the procedures used.

A study was made of the conversion of creatine into creatinine by heating in a water bath with 3,5-dinitrobenzoic acid, which then was used for the development of color *in situ*. The conversion requires heating for 3 hours with excess of the acid, and therefore offers no advantage over the well known procedure with hydrochloric acid. The extraneous color produced as a result of the heating with urine is negligible, but the excess acid dissolves slowly in sodium carbonate so that the development of color must be delayed longer than is desirable.

SUMMARY

Procedures are described for the determination of creatinine of urine and of blood with sodium 3,5-dinitrobenzoate and alkali. With this reagent the extraneous color is negligible when amounts of creatinine greater than 0.02 mg. per 10 cc. are determined. The color is more easy to read in a colorimeter than that obtained in the Folin picrate procedure. The reagent is more specific toward creatinine than is picrate, and with tungstic acid blood filtrates the values are practically identical to those obtained after adsorption of the creatinine upon Lloyd's reagent.

Addendum—The authors had not seen the article by Benedict and Behre (9) on the use of 3,5-dinitrobenzoic acid for the determination of creatinine until after the proof of this paper had been corrected. Our work, therefore, should be considered as supplementing theirs.

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THE NITROGEN PARTITION IN THE URINE OF VARIOUS PRIMATES*

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In spite of the fact that primates within recent years have assumed an increasingly prominent position as laboratory animals, many phases of their physiological functions have received scant attention. Reports on the partition of the urinary nitrogen of any of the primate group are limited to the study of Hunter and Givens (41) on a green monkey and that of Wakeman and Morrell (83) on macaques.

Methods and Procedures

Animals Used—The experiments included observations on sixteen animals representing ten species (Table I). With three exceptions all were normal, unoperated animals. The gibbon, the chimpanzee Darby, and the spider monkey had made complete recoveries from neurological operations performed 2 years to 3 months previously and the lesions were not such as to influence the results of this investigation.

In order to complete the primate series, as well as for comparison of results, analyses were also made on the urine of two normal human subjects.

Feeding and Care of Animals—The feeding habits of subhuman primates make them ill adapted to the prerequisites of a metabolic experiment. Synthetic diets and food mixtures were erratically

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eaten and often scattered so badly that measurement of amounts proved impossible. Forced feeding of liquid diets was impracticable with the larger and less tractable animals. The diet was, therefore, restricted to those foods forming a fairly complete diet which were most universally accepted, banana (73), white bread, and "chimeracker" (78).

To avoid contamination of the urine by bread and cracker crumbs, limited feeding periods were employed on collection days. When there was a large variation between the quantity of bread and cracker which an animal would eat in the limited time and that consumed if the foods were available all day, two diets were

TABLE I
Classification of Animals Used for Observation

	No. of animals	Name used	Genus and species*
Old World apes and monkeys	4	Macaques	<i>Macaca mulatta (rhesus)</i>
	1	Pig-tailed monkey	" <i>nemestrina</i>
	1	Sooty mangabey	<i>Cercocebus torquatus atys</i>
	1	Tufted "	" <i>albigena</i>
	2	Baboons	<i>Papio papio</i>
	1	Gibbon	<i>Holobates lar</i>
	1	Orang-utan (Lulu)	<i>Pongo pygmaeus</i>
	3	Chimpanzees (Darby, Ghandi, Joan)	<i>Pan satyrus</i>
New World monkeys	1	Capuchin	<i>Cebus albifrons</i>
	1	Spider monkey	<i>Ateles geoffroyi</i>

* For classification see Zuckerman and Fulton (94).

fed. Diet 1 contained those amounts of food which were consistently eaten by an animal during the course of a day. Diet 2 contained only that quantity of bread and cracker which was consumed during a 1 to a 1½ hour period. To minimize the effects of such a change, Diet 1 and Diet 2 were continuously alternated in the preliminary period, as well as during the subsequent time, when collections were made every other day, coincident with the feeding of Diet 2. All foods were weighed and the diets kept as constant as possible, although variations in appetite were met with frequently in all the animals. The two students whose urine was analyzed were restricted to the same foods as the animals, the

weighed daily ration being divided into meals which were eaten at the same time every day.

The compositions of the individual diets were calculated from the formula for chimcracker (78) and from analytical data available in the Yale Laboratory of Physiological Chemistry. The diets may be considered as essentially free of creatinine, creatine, and the purines. The milk (6, 12, 17, 81) and oatmeal (4, 88) present in chimcracker give it a creatine, creatinine, purine nitrogen value of about 4 mg. for 100 gm. of cracker. Ingested nitrogen from this source amounted to less than 0.2 mg. per kilo of body weight for all subjects except the chimpanzee Ghandi who consumed 0.4 mg. per kilo.

The animals were kept in regulation metabolism cages during the experimental period. Food was given twice daily, cracker and bread being fed in the morning and banana in the afternoon. The pan of the metabolism cage was thoroughly cleaned after the cracker and bread feeding on the days when collections were made. Water was available at all times but the containers were so placed that they could not be spilled or contaminated with urine and feces. No determinations were made until the animals had been on the diet at least 1 week. 4 days were allowed for the students to reach equilibrium.

Urine Collections—The urine was collected under toluene with enough acetic acid to change the normally alkaline reaction to one which was slightly acid, thus avoiding the possible loss of ammonia. Following the procedure of Hunter and Givens (41), 48 hour collections were made in the beginning of the series; the urine was combined with cage washings and made up to 1 liter. Later, however, the necessity for modifying the diet on collection days led to the use of 24 hour specimens, collected every other day, which, with cage washings, were not diluted to more than $1\frac{1}{2}$ to 2 times the original volume. Owing to the difficulties involved in the catheterization of such a group of animals, the periods were delimited solely by the times of placing and removing the collection bottle. A fair degree of regularity in habit and the fact that the beginning of the morning activities was associated with the urination made it possible to choose a 24 hour period which began and ended with a known voiding time.

The collections of human urine were made from 8 a.m. to 8

a.m. These urines, being alkaline, were treated with acetic acid as were the animal urines, and were collected under toluene. The number of analyses made for any subject was determined by the constancy of the results obtained.

Analytical Methods—The urine samples and cage washings were measured and filtered, and the analyses begun as soon as possible. With the exception of the distillation of the nitrogen determinations, all analyses were completed the same day. The analysis of the human urine was in every respect similar to that of the other samples.

Total nitrogen was determined by the macro-Kjeldahl method ((62) Methods), *urea* and *ammonia* by the urease, aeration, and titration technique of Van Slyke and Cullen (79, 80) ((62) Methods). Both methods were controlled by frequent blank determinations and by recoveries of the usual standard solutions.

The Folin procedures (25) for total and preformed *creatinine* were used. Although the autoclave method for total creatinine is not accurate for diabetic urine ((62) Methods), tests with added glucose showed that the amount of sugar present in any of the urine samples in this series was not great enough to influence the determination.

For the determination of *uric acid* the direct colorimetric procedures of Benedict and Franke (2) ((62) Methods) or Folin (26) were employed. Although both gave satisfactory recoveries of added uric acid, it was felt that the latter offered an advantage in that the cloudiness which sometimes complicated the Benedict-Franke readings was never encountered. The Folin method was therefore used for all determinations except those on the macaques and the pig-tailed monkey. Sodium tungstate, free from phenol reagent, was secured from the Mallinckrodt Chemical Works and tested with potassium xanthate as suggested by Folin (27). On the basis of the total nitrogen content, with the urine obtained from the larger animals and the capuchin, dilutions of the same order of magnitude as those used for samples of human urine were possible. For the samples obtained from the other animals the dilutions were $\frac{1}{2}$ to $\frac{1}{3}$ as great. That the figures for uric acid obtained by the direct colorimetric procedure are correct absolute values is not claimed. The methods of Folin and of Benedict and Franke were devised and tested by their authors only for human

urine, in which the proportion of uric acid to other nitrogenous material is much higher than in most other mammals. The reagents used are not specific for uric acid, and it is possible that the figures obtained from the urines of primates may not be highly accurate. However, in macaques our figures agree closely with those of Wakeman and Morrell (83) who employed the Folin-Wu precipitation method. The uric acid figures obtained by the present direct colorimetric procedure are at least of interest in indicating species variation observed with a constant method.

Total purines were precipitated by the Krüger-Schmid (46) method with the modification suggested by Benedict and Saiki (3, 56). According to Benedict the addition of acetic acid to the urine allows the quantitative precipitation of uric acid as well as the purine bases, including hypoxanthine, xanthine, adenine, 7-methylxanthine, and theophylline. The combined precipitate of uric acid and purine bases was collected by suction on a mat of washed asbestos in a 10 cc. Gooch crucible and washed. The crucible was then transferred to a Kjeldahl flask and the total nitrogen content determined by the Kjeldahl method. The filtrate gave no further precipitate when boiled with additional reagents. Blank determinations on the reagents yielded no nitrogen.

The nitrogen of the precipitate represented the nitrogen content of the total purines present in the urine (purine bases and uric acid). The uric acid nitrogen, obtained by the Benedict-Franke or Folin methods, subtracted from the total purine nitrogen gave the value for the purine base nitrogen.

DISCUSSION

Total Nitrogen Excretion—The data for the dietary and excretory nitrogens are given in Table II. It will be seen that, although the average nitrogen per kilo of body weight ingested and eliminated by the individual tends to be lower and more variable in Group B than Group A, there is no constant relationship in either group between the total nitrogen per kilo excreted and (a) the average nitrogen per kilo ingested in the diet, (b) the nitrogen per kilo ingested on the day of the collection, (c) the fall in nitrogen intake as a result of the change from Diet 1 to Diet 2.

That the variations observed may be accounted for by factors

TABLE II
Dietary Nitrogen and Total Nitrogen Excretion

Group A represents animals ingesting constant diets; Group B, animals ingesting varying amounts of Diet 1 as Diet 2. Diet 1 consists of the food consumed on the days when no collections were made; Diet 2, the food consumed on the days when urine was collected; average diet, one-half the food consumed in a 2 day period on the alternating Diet 1, Diet 2 régime. The age symbols are I. = immature, A. = adolescent, M. = mature.

Animal	Weight	Age	Diet					Total urine N		
			Calories per kilo		N per kilo					
			Average diet	Diet 2 as per cent of Diet 1	Average diet	Diet 2	Diet 2 as per cent of Diet 1	Gm. per kilo	Per cent of Diet 2 N	Per cent of average diet N
	kg.									
Group A										
Macaque 1.....	3.7	A.	94	100	0.30	0.30	100	0.12		40
“ 2.....	3.35	“	104	100	0.33	0.33	100	0.17		51
“ 3.....	3.6	“ -M.	125	100	0.44	0.44	100	0.20		46
“ 4.....	3.2	“	118	100	0.39	0.39	100	0.17		44
Pig-tailed monkey...	4.4	“ -M.	72	100	0.23	0.23	100	0.16		70
Sooty mangabey.....	6.7	“	113	100	0.38	0.38	100	0.13		35
Minimum.....			72		0.23	0.23		0.12		35
Maximum.....			125		0.44	0.44		0.20		90
Average.....			104		0.35	0.35		0.16		48
Group B										
Tufted mangabey...	2.65	I.	87	92	0.32	0.30	88	0.12	39	34
Baboon 1.....	4.2	“	106	64	0.33	0.20	44	0.11	54	32
“ 2.....	6.45	A.	139	80	0.40	0.24	43	0.11	47	28
Gibbon.....	3.6	I.	111	56	0.31	0.15	33	0.06	42	20
Lulu.....	26.0	“ -A.	78	58	0.28	0.17	45	0.11	64	32
Ghandi.....	19.6	“	70	21	0.29	0.06	11	0.12	190	41
Darby.....	19.0	“	53	64	0.20	0.14	54	0.16	120	64
Joan.....	19.8	“	69	55	0.21	0.10	32	0.13	132	43
Capuchin.....	1.65	A.-M.	163	48	0.55	0.25	30	0.30	120	55
Spider monkey.....	3.8	M.	90	82	0.26	0.21	70	0.17	80	67
Minimum.....			53	21	0.20	0.06	11	0.06	39	20
Maximum.....			163	92	0.55	0.30	88	0.30	190	67
Average.....			97	62	0.31	0.18	45	0.14	89	42
Students										
M. R.....	56.0	M.	37	100	0.16	0.16	100	0.16		95
M. D.....	63.0	“	34	100	0.14	0.14	100	0.13		90

other than the diet (inaccurately delimited urine collections and individual characteristics of the animals) is also suggested by the results of Wakeman and Morrell (83). In experiments in which the quantitative consumption of the food was insured by the use of liquid diets administered by stomach tube, animals ingesting 0.21, 0.19, and 0.13 gm. of nitrogen per kilo excreted respectively 72, 50, and 66 per cent of the ingested nitrogen. Another animal, reported as not in very good condition, had a nitrogen intake of 0.18 gm. per kilo of which the urine nitrogen represented 102 per cent.

An abrupt fall or rise in nitrogen consumption is associated under normal conditions with a change in a similar direction of the amount of total nitrogen excreted, and a continued gradual shift of the urinary nitrogen toward equilibrium with that ingested. The effect of a constant shift from high to low nitrogen intake and the reverse, as with the daily alternation from Diet 1 to Diet 2 and back again, is questionable. It is probable that such a régime would lead to a smaller daily variation of excreted nitrogen about the mean of the two extremes resulting from the first few changes. Such an adaptation would explain why the nitrogen per kilo excretions of Group B were only slightly lower than those of Group A. On this basis a comparison of the nitrogen excretion of the two groups relative to the average diet rather than Diet 2 for Group B would seem more justified.

Although the diet is in all cases adequate by the standard of the White House Conference on Child Health and Protection (89), *i.e.* 0.24 to 0.1 gm. of nitrogen per kilo of body weight with caloric intakes of 30 to 100 calories per kilo, the animals retain considerable amounts of ingested nitrogen. Since the mature students were in apparent nitrogen equilibrium on the same diet with lower caloric and nitrogen consumptions per kilo, the nitrogen retention shown by the animals may be explained in relation to their estimated ages as similar to that observed in children (43).

Nitrogen Partition—The average results obtained from the urine analyses for each animal are given in Table III, while the percentages of the total nitrogen represented by each constituent are found in Table IV.

(a) *Urea and Ammonia*—With the exception of the baboons and Macaque 4, the percentage values for urea and ammonia are

TABLE III
Urine Analyses

Animal	Weight kg.	No. of tests	Average gm. of urine N per 24 hrs. as										
			Total N	Urea and am- monia	Urea	Am- monia	Total creati- nine	Pre- formed creati- nine	Crea- tine	Total purines	Purine bases	Uric acid	Unde- ter- mined N
Macaque 1.....	3.7	3	0.44	0.33	0.32	0.01	0.053	0.044	0.009	0.008	0.007	0.001	0.053
“ 2.....	3.35	3	0.58	0.43	0.41	0.02	0.043	0.033	0.010	0.009	0.008	0.001	0.028
“ 3.....	3.6	4	0.73	0.59	0.56	0.03	0.040	0.034	0.006	0.017	0.014	0.003	0.085
“ 4.....	3.2	4	0.69	0.53	0.42	0.11	0.032	0.028	0.004	0.011	0.010	0.001	0.075
Pig-tailed monkey.....	4.4	8	0.70	0.55	0.53	0.02	0.056	0.049	0.007	0.009	0.007	0.002	0.090
Sooty mangabey.....	6.7	4	0.87	0.63	0.59	0.04	0.098	0.090	0.008	0.013	0.008	0.004	0.066
Tufted “.....	2.65	6	0.31	0.23	0.21	0.02	0.029	0.025	0.004	0.008	0.005	0.003	0.044
Baboon 1.....	4.2	5	0.45	0.29	0.27	0.02	0.051	0.046	0.005	0.012	0.007	0.005	0.097
“ 2.....	6.45	7	0.73	0.42	0.39	0.03	0.085	0.073	0.012	0.019	0.015	0.003	0.231
Gibbon.....	3.6	5	0.22	0.16	0.16	0.00	0.020	0.018	0.002	0.008	0.005	0.003	0.014
Lulu.....	26.0	4	2.93	2.19	2.11	0.08	0.243	0.177	0.066	0.078	0.028	0.050	0.417
Ghandi.....	19.6	4	2.28	1.91	1.79	0.12	0.191	0.173	0.018	0.115	0.035	0.080	0.412
Darby.....	19.0	3	3.11	2.35	2.27	0.08	0.252	0.196	0.056	0.074	0.017	0.057	0.438
Joan.....	19.8	5	2.65	1.69	1.61	0.07	0.198	0.175	0.023	0.090	0.019	0.071	0.405
Capuchin.....	1.65	2	0.50	0.41	0.39	0.02	0.018	0.016	0.002	0.011	0.008	0.003	0.063
Spider monkey.....	3.8	5	0.64	0.51	0.49	0.02	0.035	0.030	0.005	0.017	0.007	0.010	0.034
M. R.....	56.0	3	8.78	7.76	7.39	0.34	0.399	0.384	0.015	0.147	0.021	0.126	0.483
M. D.....	63.0	2	7.99	6.80	6.45	0.35	0.438	0.407	0.023	0.158	0.024	0.135	0.575

comparable throughout the series (Table IV). In view of the apparently adequate nitrogen ingestions, the low urea and ammonia excretions and the high undetermined nitrogen may be accounted for on the basis of the vegetarian diet (5, 22-24, 72) and the nitrogen retention already mentioned (43) rather than as a result of depressed nitrogen intake. That the baboons should

TABLE IV
Urine Analyses

Animal	Gm urine N per kilo	Urine N per cent of total N as									
		Urea and ammonia	Urea	Ammonia	Total creatinine	Preformed creatinine	Creatine	Total purines	Purine bases	Uric acid	Undeter- mined N
Macaque 1	0 12	75	72	2.2	12.0	10 0	2 0	1 8	1.5	0.3	12.5
“ 2	0 17	73	70	3.0	7.5	5 7	1 8	1.6	1.3	0 3	17.7
“ 3	0.20	81	77	4.0	5.3	4 5	0 8	2.4	2 0	0 4	11.6
“ 4	0.17	78	62	16 0	4.7	4.1	0 6	1.6	1 5	0.1	15 6
Pig-tailed monkey . . .	0.16	78	75	3.3	8.0	7 0	1 0	1.3	1 0	0.3	12.9
Sooty mangabey . . .	0 13	72	67	5.1	11.7	10 6	1 1	1.6	1.1	0 5	15 3
Tufted “	0.12	72	69	6.4	10.4	9 3	1 1	2.7	1.7	1.0	16 4
Baboon 1	0.11	64	59	5.4	11 6	10 5	1 1	2 6	1 6	1 0	21 5
“ 2	0.11	57	54	3.5	12.4	10.7	1.7	2.7	2.2	0.5	15.3
Gibbon	0.06	75	73	1.8	8.8	7.9	0.9	3.7	2.2	1.5	12.8
Lulu	0.11	75	72	2.8	8.2	6 0	2.2	2.7	1.0	1.7	14.2
Ghandi	0.12	71	66	4 8	7.4	6.7	0.7	4.1	1.0	3.1	15.9
Darby	0.16	75	73	2.6	8.2	6.3	1.9	2.4	0.6	1.8	14.1
Joan	0 13	74	71	3.5	8.7	7.7	1.0	3.9	0.8	3.1	15.3
Capuchin	0.30	82	78	3.8	3.5	3.2	0.3	2.2	1.6	0.6	12.7
Spider monkey	0.17	79	76	2.6	5.5	4.7	0.8	2.6	1.1	1.5	12.9
M. R.	0.16	88	84	4.2	4.6	4.4	0.2	1.8	0.3	1.5	5.5
M. D.	0.13	85	81	4.3	5.5	5 2	0.3	2.0	0.3	1.7	6.9

show decidedly lower urea percentages than the other animals (Table IV) with dietary nitrogen and total nitrogen excretions not significantly different, may possibly be due to a species characteristic, but is more probably explained by individual variation. The one animal, Macaque 4, showing a high ammonia value is also the one animal of the series in which any pathological condition

was noted. Seemingly in good condition during the experimental period, it soon afterward developed a severe bloody diarrhea and it is likely that internal disturbance was present at the time of the experiments.

(b) *Creatinine and Creatine*—Although diet has been shown to have some influence on creatinine and creatine excretions (1, 7, 15, 16, 33, 58, 65) the conditions under which these experiments were conducted do not invalidate the conception of the constancy of the absolute amount of creatinine excreted by an individual, irrespective of the character of the creatine-creatinine-free diet consumed (23-25, 68-70). The mean of the deviations from the average of the individual determinations for creatinine nitrogen for each animal is 11 per cent, with a standard deviation of 2.8. One student, M. R., showed less than 1 per cent variation from the mean, while M. D. had an average deviation of 7 per cent. Considering the probable error involved in obtaining quantitative collections of urine, and the normal variation of 5 to 10 per cent usually assumed in the definition of "constant creatinine elimination," these figures are indicative of constancy rather than variability of the creatinine excretions in this series.

Several points seem significant in the creatinine and creatine excretions of the animals. The group consisted mainly of immature and possibly adolescent individuals, Macaque 3, the pig-tailed monkey, and the spider monkey, being the only ones probably approaching maturity. However, instead of the large creatine values which might be expected (29, 32, 33, 36, 53, 66), the majority of the animals excreted less than 20 mg. per day, which is considered indicative of a creatinuria in man (Table III). On the other hand, as seen in Table V, the creatinine nitrogen coefficients, except for that of the gibbon, are higher than the range observed for man (36) ((62) Interpretations). Since this was also true of the green monkey and the macaques studied by Hunter and Givens (41) and Wakeman and Morrell (83) respectively, the finding probably indicates a characteristically high endogenous creatinine metabolism in the subhuman primates.

The low creatinine coefficient shown by the gibbon agrees with the low metabolic rate observed for this animal by Bruhn (10). Nevertheless, it is impossible to say, on the basis of determinations made on one individual, whether or not the findings are characteristic of the species.

There is also (Table V) some suggestion of sex difference in the creatinine nitrogen coefficients of the mangabeys, baboons, and chimpanzees. In these species the coefficients for the male are higher than those for the female, but the small number of cases and the reversal of magnitude seen in the macaques preclude an

TABLE V
Creatinine Nitrogen Coefficients

Females	Mg. creatinine N per kilo excreted as			Males	Mg. creatinine N per kilo excreted as		
	Total creatinine	Preformed creatinine	Creatine		Total creatinine	Preformed creatinine	Creatine
Macaque 1.....	14.2	12.0	2.3	Macaque 4.....	9.9	8.6	1.3
" 2.....	12.9	9.8	3.1				
" 3.....	14.3	12.2	2.1				
Pig-tailed monkey.....	12.8	11.2	2.8				
Average	13.6	11.3	1.4				
Tufted mangabey.....	11.0	9.6	1.4	Sooty mangabey.....	14.6	13.3	1.3
Baboon 1.....	12.2	11.1	1.1	Baboon 2.....	13.2	11.3	1.9
Chimpanzee				Chimpanzee			
Joan.....	9.6	8.7	1.0	Ghandi.....	10.2	9.0	1.2
				Darby.....	13.3	10.2	3.0
				Average.....	11.8	9.6	2.1
Gibbon.....	5.4	4.9	0.5	Capuchin.....	10.6	9.7	0.9
Lulu.....	9.3	6.8	2.5	Spider monkey.....	9.3	8.2	1.1
Students							
M. R.....	7.1	6.9	0.2				
M. D.....	7.1	6.7	0.3				
Average.....	7.1	6.8	0.2				

assumption as to the validity of the observation. Whether or not this relationship is present in a larger group of animals is a matter which deserves further investigation. So far as I am aware, sex differences in creatinine excretion have not been reported for any species except man (36).

(c) *Uric Acid and Purines*—The results of the uric acid and purine analyses are perhaps the most interesting of the series. The percentage of the total urinary nitrogen represented by these substances (Table IV) seems to indicate a gradual increase in the amount of endogenous uric acid excreted, proceeding from the Old World monkeys to the chimpanzee, and a similar change in the two New World species. The change occurs in the opposite direction from that which would be expected if it were a function of the diet alone. Fasting and low nitrogen diets have been shown to decrease endogenous uric acid excretion, while an increase follows a rise in the amino acid, carbohydrate, or caloric value of the food (13, 34, 47–52, 64, 71, 77). In this series, ingestions of the lowest number of calories and mg. of nitrogen per kilo of body weight are associated with the largest amounts of uric acid in the urine.

A comparison of the Old World animals on the basis of uric acid excretion relative to both the total nitrogen (Table IV) and to body weight (Fig. 1) differentiates the chimpanzees from a combined group of the monkeys, baboons, and gibbon, with the orang-utan Lulu holding an intermediate position. The comparison becomes more striking when the group averages are summarized as in Table VI. It is also evident from Table VI that, although the magnitude of the total purine nitrogen excretion appears to be an individual characteristic, the proportion of the total purines represented by uric acid emphasizes the group differences established for the Old World monkeys on the basis of uric acid alone. With regard to the total purine nitrogen and total nitrogen excretion the two New World species, the capuchin and the spider monkey, may be placed respectively at the upper range of the monkey-baboon-gibbon group and with the orang-utan. On the basis of body weight, however, both have comparatively high uric acid values, comparable to that of the orang-utan.

Uric acid represents a greater proportion of the total purine nitrogen excretion of the human subjects than of any of the sub-human species, although the magnitude of the uric acid excretion is less than that of the chimpanzee, both in relation to the total urinary nitrogen and to body weight.

Extensive studies on the comparative physiology of purine metabolism have included analyses of urines obtained from several

macaques (84, 91), a green monkey (35, 38-40), a questionable cynocephalus (91) and capuchin (84), and three chimpanzees (91). The chimpanzees, on variably purine-free diets and with only approximately timed urine collections, excreted around 6 mg. of uric acid per kilo of body weight. The later analyses on the green

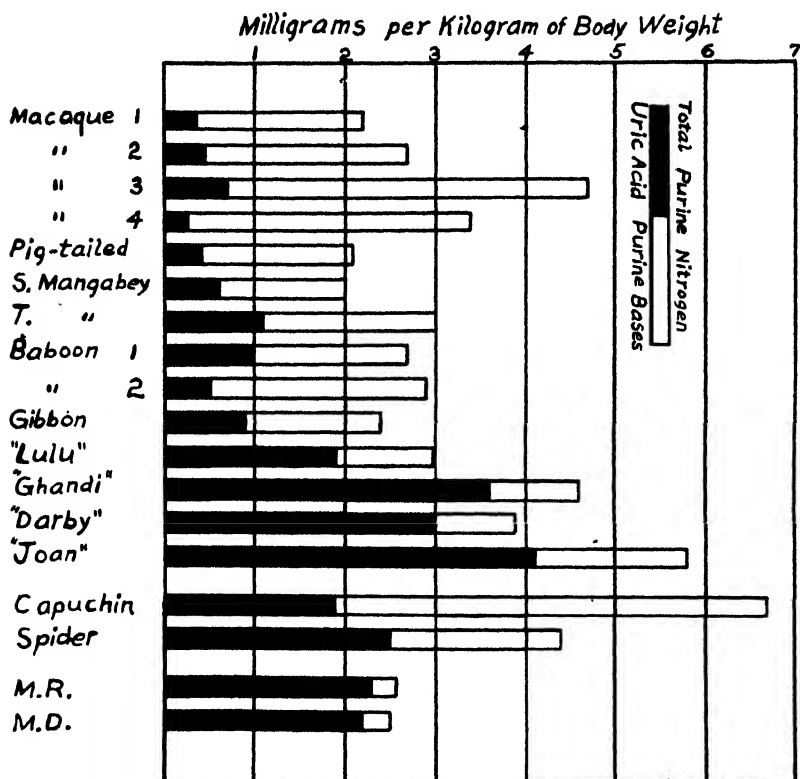


FIG. 1. Total purine and uric acid nitrogen excretions per kilo of body weight.

monkey yielded 0.34 mg. of uric acid nitrogen per kilo. With the other monkeys little or no uric acid was obtained.

These experiments, based on the assumption of the quantitative oxidation of uric acid to allantoin (in animals excreting no uric acid), were supported by the presence of a uricolytic enzyme in the monkey liver (84). In the tissues of the orang-utan and chim-

panzee no such enzyme was found (85), nor is it present in man (59, 86, 87, 90, 92).

The validity of the concept that lack of uricolysis explains the excretion of uric acid by man and the chimpanzee as opposed to the monkey and other Mammalia may be seriously questioned. The majority of evidence from the recovery of ingested or injected purine substances (11, 14, 18, 30, 31, 39, 40, 57, 67, 74-76) and the experiments of Folin, Berglund, and Derick (28) showing that uric acid may be stored in the kidney and destroyed by the blood of various mammals and man is in favor of some form of uric acid destruction in all species. Despite the absence of a uricolytic enzyme, man has been found to excrete significant amounts of allantoin associated with polyurias of various origins (19-21, 44,

TABLE VI
Purine Nitrogen

	Per cent of total N excreted as			Mg. N per kilo excreted as		
	Total purine	Purine bases	Uric acid	Total purine	Purine bases	Uric acid
Monkey group	2.2	1.6	0.6	2.5	1.9	0.6
Orang-utan.....	2.7	1.0	1.7	3.0	1.1	1.9
Chimpanzees	3.5	0.8	2.7	4.9	1.1	3.8
Man	1.9	0.3	1.6	2.6	0.4	2.2
Capuchin.....	2.2	1.6	0.6	6.7	4.8	1.9
Spider monkey.....	2.6	1.1	1.5	4.4	1.9	2.5

45). Furthermore, the monkey, whose uricolytic power lies solely in the liver (84), failed to show significant changes in blood or urinary uric acid even in the terminal stages of experimentally produced yellow fever when the rise in blood amino acids, the proportional decrease in urea, and the extreme hypoglycemia showed severe liver damage (82, 83).

In man the liver is also apparently not important in purine metabolism. Rabinowitch's (63) case of acute yellow atrophy showed the same blood picture as reported by Wakeman and Morrell (82) in the macaque with yellow fever. These results are the opposite of those obtained by Bollman and Mann (8, 9, 55) and McMaster and Drury (54) after removal of the liver in the dog and rabbit respectively, and suggest a similarity in the mech-

anism of uric acid excretion in the monkey and man as opposed to the other Mammalia.

In view of the above discussion, the validity of comparing the primates on the basis of purine metabolism, as demonstrated by uric acid or allantoin excretion, or the presence or absence of a uricolytic enzyme, may be questionable. Yet, the constancy of the endogenous uric acid and allantoin excretions in a species as a whole seems to be a characteristic so definite that Onslow (60, 61) was able to show a Mendelian ratio for the transmission of the Dalmatian coach-dog peculiarity of excreting relatively large amounts of uric acid over allantoin. Zuckerman (93) has mentioned purine metabolism as one of the physiological characters to be considered in the "functional phylogeny" of the primates. Taking his data from Hunter and Givens (35, 37, 40, 42), Wiechowski (91), and Wells (84, 85), he assumed a direct correlation between the absence of a uricolytic enzyme and uric acid as opposed to allantoin excretion. Until such a relationship is proved for the primates, it would perhaps be more advisable to consider uric acid and allantoin as separate factors, both varying from the Old World monkeys to the apes in the direction of greater similarity with man.

The New World monkeys of the series reported here excreted significant quantities of uric acid, comparable, per kilo of body weight, to that obtained from the gibbon or orang-utan. The higher excretion in the spider monkey as compared with the capuchin may, perhaps, be indicative of the development of this physiological characteristic in the *Platyrrhini* in a manner similar to that in which it developed in the *Catarrhini*, while the fact that the animals excrete uric acid in any quantity, adds one more physiological factor in which the New World resemble the Old World primates.

SUMMARY

1. A group of sixteen subhuman primates, representing eight genera and ten species, and two human subjects were studied for the partitioning of urinary nitrogen under as constant and comparable dietary conditions as possible.

2. The animals reacted similarly to children in retaining varying amounts of the apparently adequate dietary nitrogen.

3. The distribution of urea, ammonia, creatinine, and unde-

terminated nitrogen was similar to that observed by Folin with subjects on a vegetarian diet, and to that shown by children.

4. High creatinine nitrogen coefficients as compared with the normal range for man were observed in all cases except the gibbon.

5. An insignificant creatinuria was consistently present.

6. The excretion of the material determined by the direct colorimetric uric acid method, expressed as per cent of total nitrogen elimination or as a coefficient of body weight, is higher for the orang-utan and still higher for the chimpanzee than for the rest of the Old World animals as a group.

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URATE DISTRIBUTION IN BLOOD*

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In the study of the distribution of electrolytes between cells and plasma, Henderson (1), Van Slyke *et al.* (2), and others have dealt principally with ions occurring naturally in relatively large concentration; *i.e.*, bicarbonate and chloride. Other ions, as lactate and urate, are present in normal blood in such low concentration that the determination of their distribution between cells and plasma is accompanied by particular technical difficulties. There are certain pathological conditions, however, of which gout is an example, in which the urate concentration in blood is increased sufficiently to reduce some of the technical difficulties. In the blood from such patients it is possible to measure the changes in distribution of this electrolyte between serum and cells with change in pH.

In this communication there will be described certain physico-chemical properties of blood obtained from patients suffering from gout. Specifically, the applicability of the Gibbs-Donnan law of equilibrium to the distribution of urate ions in these bloods will be tested. A general description of the specimens of blood is unnecessary as no significant variation from normal human blood has been noted concerning chloride, bicarbonate, or hemoglobin content. Our present purpose is the consideration of the concentration of urate in cells and plasma as a function of hydrogen ion concentration and degree of oxygenation of hemoglobin.

Material and Methods—The three gouty patients in this study from whom blood was obtained were suffering from recurrent attacks of acute gout. The diagnosis in patients F. M. (3) and

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F. N. was based upon the recovery and identification of sodium urate crystals from cutaneous tophi. Patient M. T. had clinical gout, but at the time this work was done had developed no palpable tophi.

Venous blood was drawn from these patients in the basal state and immediately defibrinated. Approximately 15 cc. portions of this blood were added to tonometers at varying tensions of CO_2 and O_2 and were equilibrated in a water bath at 37.5° (3). After 20 minutes of equilibration, whole blood for measurement of oxygen content, carbon dioxide content, and urate concentration was removed. The remaining blood was transferred under oil to a graduated centrifuge tube and centrifuged for three 20 minute periods. Calculation of the cell volume at infinite time was made according to the method of Hirota (4). Values for pH_s were derived from the equation:

$$\text{pH}_s = \text{pK}'_s + \log(\text{BHC}\text{O}_3)_s - \log(\text{H}_2\text{C}\text{O}_3)_s$$

Urates were determined in whole blood, serum, and cells according to the method of Benedict and Behre (5) or that of Folin (6). In the experiment on January 28, 1936, the urate concentrations were determined by both methods on aliquot portions of the same sample. The accuracy of the Benedict determination on aliquot portions of similar sera on subsequent days was investigated in seventeen samples obtained from as many patients with various pathological conditions. The urate concentration was determined first on each sample on the day when the blood was withdrawn. After the remainder of each sample had been kept at 4° for a period varying between 2 and 6 days, the determination was repeated. In twelve of the sera the maximum variation was less than 0.02 milli-equivalent per liter. In the remaining five samples the maximal variation was less than 0.04 milli-equivalent per liter.

Samples of sera measured volumetrically and weighed samples of cells were used for determination of urate and chloride. The distribution between serum and cells was calculated according to the equations:

$$\frac{(\text{U}')_c/(\text{H}_2\text{O})_c}{(\text{U}')_s/(\text{H}_2\text{O})_s} \text{ and } \frac{(\text{Cl}')_c/(\text{H}_2\text{O})_c}{(\text{Cl}')_s/(\text{H}_2\text{O})_s}$$

In these equations the concentration of the urate ion (U') and of the chloride ion (Cl') are expressed in milli-equivalents per liter. H_2O was determined on weighed samples.¹

In certain experiments the blood was treated as described above and without the addition of any substance. In other experiments from 0.5 to 0.6 milli-equivalent per liter of unneutralized uric acid was added to the whole blood. All of the uric acid which was added did not go into solution; therefore, it was necessary to filter these bloods through several layers of fine gauze before equilibration.

Natural Occurrence of Urate within Cell—It is assumed generally that urates are present naturally in blood cells as well as serum (7, 8) and that the erythrocyte wall exercises a conditioned permeability in respect to the transfer of this ion (9). In view of Olmsted's recent report (10) that glucose is not present in human blood cells immediately after withdrawal from the body, it was thought desirable to carry out similar experiments concerning the presence of urate within the cell. For this purpose blood was drawn from patient F. N., placed in a centrifuge tube under oil, and centrifuged at once. No anticoagulant or other substance was added. The elapsed time between the withdrawal of the blood and the precipitation of the serum protein was approximately 10 minutes. Urate was determined shortly after by the method of Benedict. Other samples from the same venesection were allowed to stand for 2 hours at 4° and 37.5° respectively, before analysis. In a similar fashion samples were kept at 4° and 37.5° respectively, for 6 hours before analysis. At the end of 2 hours there was a decrease in $(U)_c$ from 0.50 to 0.49 milli-equivalent per liter and at the end of 6 hours it had decreased to 0.48. No difference in urate distribution was observed between the samples kept cold and those kept warm. In another experiment done on the same day, $(U)_c$ and $(U)_s$ were determined as well as $(U)_w$. Blood from the same venesection as above was defibrinated under oil and portions were equilibrated at $pH_s = 7.30$ for 15, 60, and 120 minutes respectively, in the water bath at 37.5°. There was a decrease in $(U)_c$ from 0.50 to 0.48 milli-equivalent per liter over the 2 hour period. The $(U)_c$ remained unchanged at 0.21 and $(U)_s$

¹ In this paper the subscripts c , s , and b refer to cells, serum, and whole blood respectively.

TABLE I
Experimental Observations on Oxygenated Blood

Date	Subject	Uric acid method	Whole blood					Serum				Cells		
			pCO ₂	Total CO ₂	Oxygen capacity	Cell volume	Urate	Bicarbonate	Chloride	Urate	Water	pH	Chloride	Urate
			mm. Hg	m.-eq. per l.	m.-eq. per l.	per cent	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	m.-eq. per l.	gm. per l.		m.-eq. per l.	m.-eq. per l.
Jan., 1936														
21	M. T.	Folin	10.1	8.1	6.97	37.1	0.42	9.3	114.7	0.51	935	7.58		0.22
			181.7	28.5		41.5	0.45	25.0		0.45		6.75		0.47
23	"	Benedict	8.4	7.0	7.17	38.4	0.37	7.8	116.4	0.64	935	7.58	58.1	0.23
			174.3	29.4		41.0	0.49	24.9	109.8	0.58	932	6.77	75.9	0.49
28	F. N.	"	13.7	10.3	10.5	51.5	0.47	13.2	109.1	0.66	935	7.60	53.7	0.26
			218.0	38.3		55.0	0.51	35.7	99.5	0.61	931	6.83	62.2	0.31
28	"	Folin*					0.56			0.83				0.46
							0.57			0.74				0.47
30	F. M.	Benedict	13.0	11.7	10.8	51.0	0.56	15.1	104.6	0.89	939	7.68	43.8	0.27
			219.2	40.7		53.4	0.55	39.0	92.0	0.69	936	6.87	53.7	0.39

* Determinations were made on aliquot portions of the same filtrates.

decreased from 0.32 to 0.30 milli-equivalent per liter during the same period. These data are interpreted as indicating that within the time limits of our experiment at a constant pH, there is no significant migration of urate from serum to cells and that urate does exist in cells *in vivo*.

Change in $(U)_s$ and $(U)_c$ As a Function of pH_s —In Table I are given data from four experiments on oxygenated blood to which no uric acid was added. In all experiments the reaction of the drawn blood was altered to "give one set of determinations in the alkaline range near $pH_s = 7.60$ and another set of determinations in the acid range near $pH_s = 6.80$. This range of hydrogen ion concentration exceeds greatly the usual physiological variation. Associated with this change in pH_s were the well recognized changes in cell volume and in (HCO_3) and (Cl) distribution. The average change in pH_s for the four experiments shown in Table I was 0.81. The average decrease in $(Cl)_s$ was 9.6 and the average increase in $(Cl)_c$ was 8.8 milli-equivalents per liter. The average increase in cell volume percentage was 3.2. The change in $(U)_s$ was in the same direction as, and was roughly proportional to, the change in $(Cl)_s$. The data in Tables I and II are not corrected for the increase in free uric acid in the acid range. In an aqueous solution of pH 7.60 about 99 per cent of the total urate in solution is in the form of sodium urate and only 1 per cent as free acid (11). At pH 6.80 about 84 per cent is in the form of the sodium salt and 14 per cent as free acid. When these corrections are applied to the data the percentile shift is increased. Simultaneous with the decrease in $(U)_s$ there was an increase in $(U)_c$. This change is similar to that observed for $(Cl)_c$. There was no change in $(U)_i$ except in the experiment of January 23, 1936; the discrepancy of 0.12 milli-equivalent in that experiment presumably was due to an error in determination. It is evident from these data that there is a migration of the urate ion between serum and cells with varying pH and that this migration is like that observed for the chloride ion.

Distribution of Urate between Cells and Plasma—This has been a subject for dispute (12, 13) since the introduction into clinical chemistry of micromethods for the determination of this constituent in human blood. It is generally accepted that the concentration of urate in exudates and edema fluid is similar to the

TABLE II
Experimental Observations on Oxygenated Blood with Added Uric Acid

Date	Subject	Uric acid method	Whole blood				Serum				Cells			Chloride r	Urate r	
			pCO ₂	Total CO ₂	Cell volume	Urate	Bicar-bonate	Chloride	Urate	Water	pH	Chloride	Urate			Water
Jan., 1936																
21	M. T.	Folin	8.8	7.8	37.7	0.98	9.0	114.6	1.24	934	7.62	60.3	0.56	782	0.63	0.54
			207.5	30.3	42.5	1.08	26.2	106.9	1.10	934	6.71	76.0	0.88	796	0.83	0.94
23	"	Benedict	9.0	6.8	38.8	0.74	7.7	116.9	1.03	934	7.55	63.6	0.46	777	0.66	0.53
			185.4	29.1	41.2	0.78	25.0	109.0	0.95	930	6.75	75.5	0.63	789	0.82	0.78
28	F. N.	"	11.9	9.5	51.5	0.87	11.9	102.1	1.20	939	7.61	52.1	0.47	769	0.62	0.48
			215.4	36.9	54.6	0.89	34.4	92.6	1.08	936	6.82	65.2	0.67	796	0.83	0.75
28	"	Folin*				1.16			1.63				0.56			0.42
						1.08			1.30				0.83			0.75
30	F. M.	Benedict	11.7	11.0	51.2	1.06	14.1	104.8	1.47	940	7.70	43.5	0.63	749	0.53	0.53
			220.6	40.7	53.3	0.90	37.8	93.6	1.14	935	6.85	59.6	0.72	763	0.79	0.77

* Determinations were made on aliquot portions of the same filtrates.

concentration in serum (14), but no such agreement exists concerning the distribution in blood cells and serum. The ratio 23:100, not corrected for water content, given by Folin and Svedberg (15) is the lowest of any large series in the literature. When Benedict's ratio of 74:100 (5) is corrected for water content of serum and cells, a ratio of approximate unity is obtained.

In Table II are given the experimental data on blood to which uric acid was added. Except for the addition of this substance to the blood the experiments were performed in the same manner as those depicted in Table I. Our principal interest in these

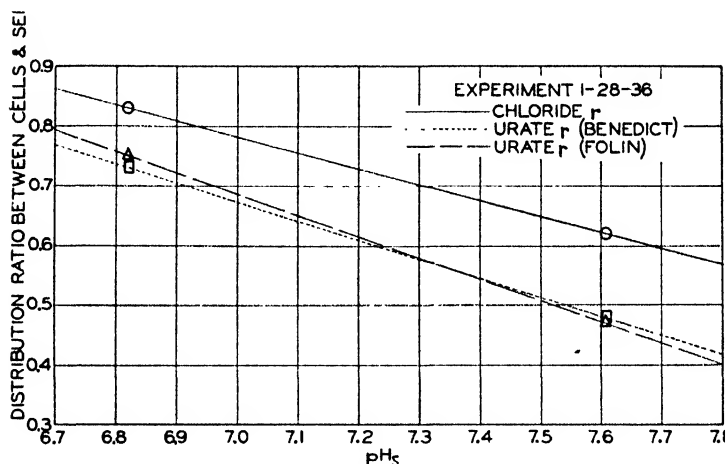


FIG. 1. Distribution ratio of chloride and urate as a function of pH_s.

experiments was the changing distribution ratio as a function of pH_s. In Tables I and II and Figs. 1 and 2 the values for (U)_c are related to the pH of the serum and not of the cell. If the ratio is calculated for pH within the cell and the data corrected accordingly, the urate r in the acid range is slightly greater than shown.

In these experiments it is presumed that the added uric acid distributed itself between the serum and cells and behaved in a fashion similar to the urate already present. The percentile increase in concentration in serum and cells substantiates this presumption. With increasing acidity (Fig. 1) the urate distribution ratio increased at approximately the same rate as the chloride

ratio. At $\text{pH}_s = 7.40$ the average chloride ratio of the three experiments was 0.68. At the same pH_s , the average urate distribution ratio was 0.60. Van Slyke, Hastings, Murray, and Sendroy (16) have shown by electrometric measurements that the distribution of hydrogen ions between serum and cells is such that the ratio $(\alpha\text{H}^+)_{\text{s}}/(\alpha\text{H}^+)_{\text{c}}$ approximately equals 0.50 at $\text{pH}_s = 7.40$. The agreement of the urate ratio with the reciprocal of the hydrogen ion ratio is better than the ionic distribution of chloride and bicarbonate and suggests that the activity and the concentration of the urate ions are more nearly alike than in the case of chloride and bicarbonate.

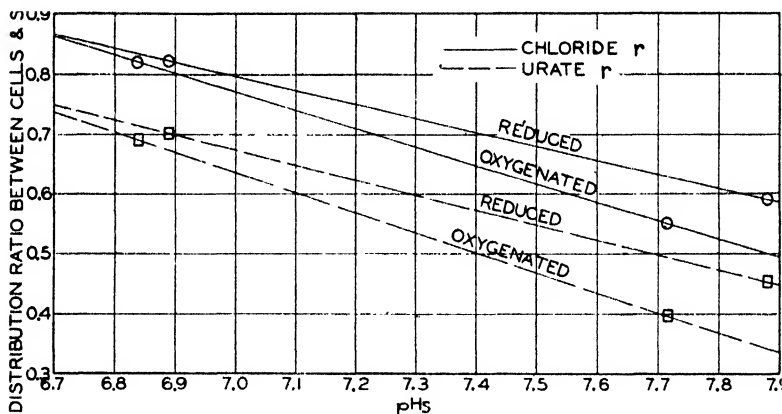


FIG. 2. Distribution ratio of chloride and urate as a function of degree of oxygenation of hemoglobin and pH_s .

Effect of Oxygenation-Reduction and pH_s Change on Urate Distribution—One experiment was performed to determine the effect of oxygenation and reduction of the blood in the acid and alkaline range on the distribution of urate between serum and cells. The data are shown graphically in Fig. 2. The anion distribution ratios predicated by Van Slyke, Wu, and McLean (2) were lower in oxygenated blood than reduced blood at the same pH_s . In our experiments at $\text{pH}_s = 7.80$ the spread of the chloride ratio between oxygenated and reduced whole blood was 0.08. At the same pH_s the spread of the urate ratio was 0.10. With increasing acidity

the spread is diminished as observed by Dill (17) for the bicarbonate distribution ratio. At $\text{pH}_s = 6.80$ the spread of the chloride ratio was 0.01 and the urate ratio was 0.02. At pH_s of approximately 6.60 the reduced and oxygenated distribution ratios for chloride and urate, respectively, are apparently equal.

Maximum Concentration of Urate in Serum—In equilibration experiments of serum with sodium urate, Gudzent (18) approached a maximum solubility with a urate concentration of 0.5 milli-equivalent per liter. Our observations indicate that at least twice this concentration may be observed in the serum from gouty patients. The maximum concentration of 0.89 milli-equivalent per liter of serum in blood drawn from M. T. on January 30, 1936, is less than 1.3 milli-equivalents per liter, the maximum solubility observed by Bechhold and Ziegler (19). In the experiment of January 28, 1936, uric acid was added to the blood and a concentration of 1.63 milli-equivalents per liter of serum was obtained. These observations suggest that Gudzent's maximum values are too low, either for urate occurring in the serum of patients with gout or for *in vitro* experiments when uric acid is added to blood.

SUMMARY

1. The concentration of urate in serum and cells agrees approximately with the Gibbs-Donnan law of equilibrium as applied by Van Slyke and associates to blood. As the reaction of the serum becomes more acid, there is a migration of urate ions from the serum to the cells. A reverse migration takes place as the reaction becomes more alkaline.

2. When urate is added to blood it distributes itself between cells and serum in the same proportion as the naturally occurring urate.

3. At $\text{pH}_s = 7.40$ the urate distribution ratio as defined by the relation

$$\frac{\text{Cell urate}}{\text{Cell water}} \div \frac{\text{serum urate}}{\text{serum water}}$$

is approximately 0.60.

4. If blood containing added urate is oxygenated or reduced, the urate ratio changes in a manner similar to the chloride and bicarbonate ratios.

5. After the addition of uric acid the maximum concentration of serum urate observed by us in one instance was 1.63 milliequivalents per liter, a value which considerably exceeds the value previously ascribed to the solubility of urate in serum.

The authors wish to acknowledge assistance from Dr. A. Baird Hastings during this investigation.

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THE RELATION OF SERUM PHOSPHATES TO PARATHYROID TETANY*

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As early as 1911 Greenwald (1) showed that one of the first detectable results of the removal of the parathyroid glands was a decreased excretion of phosphorus in the urine. In 1913 he (2) extended these studies to show that the effect was on phosphorus directly and not on sodium or potassium. Some years later Greenwald (3) again drew attention to these findings. However, little emphasis was given to phosphorus in its relation to the function of the parathyroid glands until the work of Albright, Bauer, Ropes, and Aub. In 1929 they (4) presented evidence to indicate that the primary action of the parathyroid hormone is on renal excretion of phosphorus. In a later publication Albright and Ellsworth (5) expressed the belief that the whole effect of the parathyroid secretion on the calcium and phosphorus levels of the blood could be explained on the basis of a variation in the ability of the kidney to excrete phosphorus. They believe that in hypoparathyroidism there is a retention of phosphate which forces the concentration of calcium down. The reverse is true during hyperparathyroidism, in which there is a loss of phosphorus with a subsequent solution of calcium phosphate from the skeleton. Albright, Bauer, Claffin, and Cockrill (6) are of the opinion that there is a definite renal threshold for phosphorus; in hypoparathyroidism the threshold is raised and in hyperparathyroidism it is lowered. This idea has been further developed by Ellsworth (7).

* A report concerning this work was presented before the Thirtieth meeting of the American Society of Biological Chemists at Washington, March 25-28, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, liv (1936); *J. Biol. Chem.*, **114** (1936)).

About 2 years ago the present author (8) showed that if young rats were parathyroidectomized and then placed on a high calcium-low phosphorus rachitogenic ration, the animals did not show the low serum calcium and high phosphorus characteristic of parathyroid deficiency. Instead, the blood picture was that typical of rachitic rats; that is, low blood phosphorus and normal or slightly elevated calcium. If, however, the calcium was omitted from this diet, the rats invariably went into parathyroid tetany and showed a low serum calcium and high phosphorus within a day or two after removal of the glands. The most plausible interpretation of this difference is that the high calcium intake on the one diet was sufficient to prevent the development of the hypocalcemia necessary for the onset of tetany; whereas, on the other diet the supply of calcium was inadequate to maintain a concentration of serum calcium above the tetany level. In view of the work concerning the effect of phosphorus on the development of parathyroid tetany, it is possible that the results were not due directly to the difference in calcium intake but indirectly to decreased availability of the phosphorus of the high calcium diet consequent to the formation of insoluble calcium phosphate. It may well be that the formation of an insoluble phosphate in the intestinal tract prevented the absorption and subsequent accumulation of phosphorus in the blood, with the result that the calcium remained high. If the high calcium of the diet on which tetany did not develop functioned primarily by decreasing the availability of phosphorus, it should be possible to obtain similar results with diets low in calcium but in which the availability of the phosphorus was decreased by the addition of some metal other than calcium which forms an insoluble phosphate. The results of such experiments are reported at the present time. Deobald and Elvehjem (9) have recently shown that the feeding of either aluminum or iron salts, in rather large quantities, produces a condition of phosphorus want, presumably by the formation of insoluble phosphates of these elements in the intestinal tract. In view of these observations we have used basic aluminum acetate in most of the work. A few results were also obtained with aluminum sulfate.

EXPERIMENTAL

Three diets with varying degrees of calcium deficiency were employed in these studies. In the first few experiments young rats

were fed the dry portion of the Steenbock (10) stock diet from which the 0.5 per cent calcium carbonate had been omitted and to which was added 4 per cent aluminum acetate (Diet 1). If this diet is given without the aluminum acetate, parathyroidectomized rats will regularly develop tetany with a low serum calcium and high phosphorus within 24 to 48 hours. In the present experiments the diet containing aluminum was fed for 2 to 3 weeks before the removal of the glands, during which time the animals developed rachitic symptoms. The parathyroid glands were then removed, but only those animals from which both the glands were definitely identified in the manner previously described (11) were included in the experimental results. Following parathyroidec-

TABLE I

Effect of Basic Aluminum Acetate on Parathyroidectomized Rats Receiving a Diet Moderately Deficient in Calcium (Diet 1)

Rat No.	No. of days after parathyroidectomy	Serum		Rat No.	No. of days after parathyroidectomy	Serum	
		Calcium	Phosphorus			Calcium	Phosphorus
		mg. per 100 cc.	mg. per 100 cc.			mg. per 100 cc.	mg. per 100 cc.
1, 2	Control	11.5	2.7	9, 10	Control	13.6	2.9
3, 4	2	11.2	2.3	11, 12	1	12.8	2.9
5, 6	2	11.8	2.5	13, 14	7	-13.0	3.9
7, 8	4	11.9	2.8	15	13	12.0	2.6
				16	13	11.2	3.0

tomy the animals were killed at varying intervals, and the sera were analyzed for calcium by the method of Clark and Collip (12) and for phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg (13). It was necessary to pool the blood of two or more animals to obtain sufficient serum for these analyses. In Table I are shown the results of two such experiments. In the first experiment (Rats 1 to 8) two control animals (Rats 1 and 2, sacrificed shortly before the parathyroids were removed from the others) had a serum calcium content of 11.5 and a phosphorus content of 2.7 mg. per cent. As seen from Table I, the removal of the parathyroid glands had no effect on the blood calcium or phosphorus at 2 and 4 day intervals.

In another experiment on this same diet (Rats 9 to 16, Table I)

the animals were sacrificed at intervals of 1, 7, and 13 days, with no appreciable change in the calcium and phosphorus levels of the blood. This particular group of animals had a rather high concentration of calcium in the serum. We have observed this occasionally at other times when the aluminum is given with a diet in which the calcium is either ample or only moderately deficient. None of these animals was ever observed showing any signs of tetany.

In the next experiment a diet still lower in calcium was used. A number of young rats were fed the aluminum-containing diet described above (Diet 1) for a period of 2 weeks. About 48 hours before removal of the parathyroids the animals were transferred

TABLE II

Effect of Basic Aluminum Acetate on Parathyroidectomized Rats Receiving a Diet Decidedly Deficient in Calcium (Diet 2)

Rat No.	No. of days after parathyroidectomy	Serum		Rat No.	No. of days after parathyroidectomy	Serum	
		Calcium	Phosphorus			Calcium	Phosphorus
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>			<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
17, 18	Control	11.8	2.6	27, 28	4	9.0	1.9
19, 20	1	10.4	2.7	29, 30	7	8.2	1.5
21, 22	1	9.3	Lost	31, 32	11	8.8	1.2
23, 24	2	9.3	2.1	33, 34, 35	9	4.6	13.9
25, 26	2	9.1	2.2				

to the Steenbock-Black (14) rachitogenic ration from which the calcium carbonate was omitted and to which was added 4 per cent of aluminum acetate (Diet 2). This diet contained only from 0.03 per cent to 0.04 per cent calcium. When fed without the aluminum acetate to parathyroidectomized rats, it invariably produced marked tetany, within a day or so after removal of the glands, with the usual high blood phosphorus and low calcium.

The results of this experiment are summarized in Table II. Consideration of the last group of three animals being omitted for the present, it is seen that the aluminum salt not only prevented a rise in phosphorus, but there appears to have been an actual decrease during the 11 day period following the removal of the glands. This occurred in spite of the initial low phosphorus.

There was also a definite fall in calcium but not to the level which usually results in tetany. It cannot be stated at the present time whether this drop was due entirely to the low intake of calcium or to a deficiency of parathyroid function. However, it is evident that it was not caused by a rise in phosphate.

It so happened that three of the animals of this group (Rats 33, 34, and 35, Table II) were in tetany on the 9th day following the operation. Food consumption records showed that these animals had been eating considerably less than average, and during the 24 hours previous to the onset of the tetany they had consumed practically no food. They were losing weight rapidly. As indicated in Table II, the blood phosphorus was very high and the calcium low. It appears that the inorganic phosphorus which was

TABLE III

Effect of Basic Aluminum Acetate on Parathyroidectomized Rats Receiving a Diet Containing Only Traces of Calcium (Diet 3)

Rat No.	No. of days after parathyroidectomy	Serum		Rat No.	No. of days after parathyroidectomy	Serum	
		Calcium	Phosphorus			Calcium	Phosphorus
		mg. per 100 cc.	mg. per 100 cc.			mg. per 100 cc.	mg. per 100 cc.
36, 37	Control	11.5	4.0	41, 42	2	11.1	5.0
38, 39	1	9.8	5.7	43, 44	4	11.2	3.0
40	2	11.7	4.5	45, 46	7	11.2	4.4

liberated as the result of excessive endogenous metabolism accumulated in the blood stream and forced the calcium down with the onset of tetany. Three other animals of this series died at varying intervals following the removal of the parathyroids. As they all died during the night, no tetany was observed, but it was probably experienced shortly before death.

In another series of experiments, the results of which are shown in Table III, rats were treated the same as above but were transferred to a synthetic diet (Diet 3) practically free from calcium. This diet was also low in phosphorus and contained 4 per cent of the aluminum acetate. It was the same as the low calcium diet previously described (15) except that the phosphates of the salt mixture were omitted and cod liver oil was substituted for the

carotene solution. The amount of calcium in this diet was less than 0.005 per cent. Although the intake of calcium on this diet was negligible, the calcium of the serum remained high and the phosphorus low. Despite the lower amount of calcium in the food both the calcium and phosphorus of the serum of the animals on Diet 3 were slightly higher than in the serum of the animals on Diet 2. No explanation for these somewhat paradoxical results is at present available.

Several adult rats also were fed Diet 3 for a few days, after which the parathyroids were removed. Another small group of adult rats was given a diet which was the same except that the aluminum acetate was replaced by an equal amount of crystalline aluminum sulfate. Parathyroidectomies likewise were performed a few days after the beginning of the feeding of this diet. In both these cases the calcium of the serum remained high and the phosphorus low. One adult female on the diet containing aluminum acetate gave birth to eight young 2 days after the parathyroid glands were removed. Seven of the young were still alive on the 18th day after parturition. They were all very small (average weight 10 gm.) and weak, but were covered with hair, had their eyes open, and could walk. They were considerably deformed. By the 23rd day after birth they had all died. In the absence of the parathyroid glands, with only a trace of calcium in the diet and most of the phosphorus not available, this female rat had been able to call upon its own body stores in sufficient amount to maintain itself and also keep a litter of young alive for 3 weeks. At the end of this time both the calcium and phosphorus of the serum of the mother rat were within normal limits. Owing to the fact that the food consumption of this animal had decreased considerably during the last 48 hours before it was sacrificed, the serum phosphorus was probably higher than it would have been otherwise.

In order to study further the influence of food consumption on the development of tetany under these conditions, the following experiment was carried out. A group of young rats was placed on Diet 1 and after a period of 2 weeks the parathyroids were removed. At the same time these animals were deprived of food. Without exception every animal was in violent tetany on the morning following the ablation of the glands. The blood calcium was low and the phosphorus was high. As these animals were some-

what rachitic, it was to be expected that they would develop tetany when deprived of food. However, the severity of the tetany and the regularity with which it appeared were much more marked in the animals from which the parathyroids had been removed than in a similar group which was only fasted.

Experiments were also conducted in which the aluminum acetate was omitted from the diet at the time of the removal of the glands. All of these animals developed tetany within 24 hours following the loss of the parathyroids. As in the fasting experiments, the simple omission of the aluminum acetate from the diet produced tetany in some of the animals. Here again, however, the frequency and severity of the tetany were considerably increased by removal of the parathyroids.

DISCUSSION

These data apparently are in accord with the theory of Albright and Ellsworth (5). However, it is difficult to believe that a simple decrease in the inorganic phosphorus of the blood can produce the marked degree of hypercalcemia which follows the administration of large doses of parathyroid extract. A very low concentration of phosphorus in the blood is often encountered with a normal or even a low level of calcium. When young rats, for instance, are put on a high calcium-low phosphorus rachitogenic diet the inorganic phosphorus of the blood is low, while the calcium is either normal or only slightly raised. This is the case in spite of the large amount of calcium in the food. A similar blood picture is observed in rickets produced by adding to the diet some metal other than calcium which forms an insoluble phosphate. As previously stated, during the latter type of rickets in rats the blood calcium may be a little above normal, but the rise is not more than 1 or 2 mg. per 100 cc. of serum. This is much less than the corresponding drop in phosphorus; consequently there is a fall in the calcium \times phosphorus product. Under similar conditions in the dog (16) there is no appreciable rise in the calcium of the serum. It might be pointed out, however, that as these animals were young and frequently rachitic, the stores of calcium in the bones might have been insufficient to compensate for the decrease in phosphorus. However, we have been able to produce a very low serum phosphorus in adult rats by adding aluminum sulfate to an adequate diet without any rise in serum calcium.

As further evidence in favor of the phosphorus excretion theory Shelling (17) has shown that a high intake of phosphorus predisposes to tetany in parathyroidectomized rats. Tweedy, Templeton, and McJunkin (18) have recently reported that deletion of kidney function protects the dog from overdosage effects of parathyroid extract, and have concluded that the mobilization of calcium stores of the body into the blood by parathyroid hormone is dependent on kidney function. In contrast to this, Collip, Pugsley, Selye, and Thomson (19) claim that the characteristic effect of parathyroid extract on the bones of the rat is obtainable after bilateral nephrectomy. Ellsworth and Fitcher (20) also were able to produce a marked hypercalcemia by administering parathyroid extract to dogs from which the kidneys had been removed. Goadby and Stacey (21) found that large doses of parathyroid extract did not produce the usual diuresis in patients with kidney disease, but there was a definite rise in serum calcium. They conclude that the hormone acts on the excretion of phosphorus and also to liberate calcium. From the data available at the present time this appears to be a logical view.

SUMMARY

The addition of 4 per cent of basic aluminum acetate to diets low in calcium protected rats from the symptoms which usually follow the removal of the parathyroid glands. The calcium of the serum remained high and the phosphorus low. This was found to be the case on a diet which did not contain over 0.005 per cent of calcium. If for any reason the animals did not eat after the loss of parathyroid function, serum phosphorus increased, the calcium fell, and tetany followed. Apparently, if the phosphorus of the blood can be kept low, the calcium remains above the tetany level, even though there is a pronounced deficiency of calcium in the diet. If, however, there is a source of available phosphorus either from the diet or from excessive endogenous metabolism resulting from fasting, the blood phosphorus rises, the calcium falls, and tetany appears.

The relation of these observations to the phosphorus excretion theory of parathyroid function has been discussed.

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ESTIMATION OF SMALL AMOUNTS OF CHOLESTEROL AS THE PYRIDINE CHOLESTERYL SULFATE*

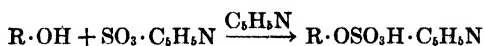
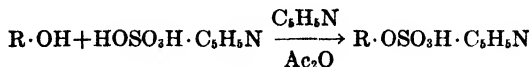
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The determination of free cholesterol in the presence of its esters in biologic materials seems to be of the utmost importance. The hitherto available methods depend upon the isolation of the digitonide and subsequent gravimetric, colorimetric, gasometric, or titrimetric estimation. However, the chemical reaction which takes place between cholesterol and digitonin is still obscure. It is, therefore, hardly surprising that various samples of digitonin may give different results. The isolation of cholesterol by digitonin in the presence of other lipids is not always quantitative (1). Digitonin affects the Liebermann-Burchard colorimetric reaction and therefore special precautions are necessary for the colorimetric estimation (2, 3).

In the present method a new chemical principle is introduced for the isolation of free cholesterol. This depends upon the insolubility of pyridine cholesteryl sulfate in petroleum ether. The amount of cholesterol in the precipitate is estimated by the more specific Liebermann-Burchard reaction. The reactions used for the preparation of the pyridine salt are illustrated in the equations below, where R represents the steryl radical.

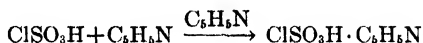


* Presented before the American Society of Biological Chemists at Washington, March 25-28, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, xcvi; *J. Biol. Chem.*, **114** (1936)).

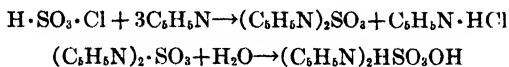
From these equations it can be seen that the reactions depend upon the presence of a hydroxyl group in the sterol.

The above reactions may be carried out in benzene, pyridine, or chloroform. Benzene was found to be most advantageous with the lipid extracts because, while the lipids are very soluble in this medium, the pyridine cholesteryl sulfate may be quantitatively precipitated by means of petroleum ether (35–60°) in which all the unchanged lipids are also very soluble. The excess precipitating agent is insoluble in the benzene-petroleum ether mixture, but this does not matter, since no interference with the Liebermann-Burchard reaction is experienced. The washings may be used for the subsequent determination of cholesterol esters.

The reagent used at first in these reactions was the solid precipitate which forms when chlorosulfonic acid is added to an excess of pyridine in chloroform with cooling. It was expected that this would be the pyridine chlorosulfonate, as illustrated in the following formula.



Upon analysis, however, this precipitate was observed to have 1 to 2 per cent of chlorine and was shown to be mostly a mixture of pyridine sulfur trioxide and pyridine sulfate, while the solution contained mostly pyridine hydrochloride. The reaction is probably the following.



To find out which part of the reagent was responsible for the reaction both pure pyridine sulfate and pyridine sulfur trioxide were tried¹ with anhydrous precautions. Pyridine sulfur trioxide formed a cholesteryl pyridine sulfate derivative readily in the

¹ The pyridine sulfate was prepared by the addition of 1 mole of sulfuric acid to 3 moles of pyridine in chloroform. The precipitated pyridine sulfate was then dried in a desiccator. Calculated for $(\text{C}_5\text{H}_5\text{N})_2 \cdot \text{H}_2\text{SO}_4$, S 12.6 per cent; found, 12.1 per cent. The pyridine sulfur trioxide was prepared by the addition of 1 mole of solid sulfur trioxide to 3 moles of pyridine in chloroform with cooling. The reaction is quite violent. Consequently, the reagent must be added slowly with constant stirring. Calculated for $(\text{C}_5\text{H}_5\text{N})_2 \cdot \text{SO}_3$, S 13.4 per cent; found, 13.1 per cent.

presence of pyridine in benzene, while pyridine sulfate did not react at all unless an internal dehydrating agent such as acetic anhydride was also used in the reaction mixture. The precipitating reagent formed by the interaction of chlorosulfonic acid and pyridine brought the reaction to about 60 per cent completion in the absence of acetic anhydride. This behavior is intermediate between that of pure pyridine sulfur trioxide and pyridine sulfate. In all of the results reported in this paper, this reagent was used in conjunction with pyridine and acetic anhydride. However, we have observed that either pure pyridine sulfate or pure pyridine sulfur trioxide may be used interchangeably in the presence of acetic anhydride.

The reaction, in the presence of acetic anhydride, goes to completion even in the presence of small traces of water or alcohol which might otherwise seriously interfere in the microprocedure. No acetylation of cholesterol at the hydroxyl group was observed under the conditions employed.

This method has several advantages over the digitonin procedure for the separation of free and combined cholesterol in the lipid extracts. The procedure is simple, accurate, and quantitative in the presence of other lipids. The determination is based upon a known chemical reaction. The reagent is stable, inexpensive, and easy to prepare. No interference with the colorimetric estimation is observed. Moreover, both free cholesterol and combined cholesterol may be determined on the same sample.

Procedure

Reagents—

Pyridine. The usual c.p. grade of pyridine is redistilled over potassium hydroxide.

Chloroform. c.p. grade dried over anhydrous calcium chloride.

Sulfuric acid. A c.p. grade of specific gravity 1.84 was used.

Chlorosulfonic acid. Technical chlorosulfonic acid is distilled in an all-glass apparatus. This precaution may not be necessary with a purer grade of reagent.

Precipitating reagent. 10 cc. of pyridine are dissolved in 20 cc. of chloroform. The mixture is cooled in an ice bath and 4 cc. of chlorosulfonic acid are added drop by drop with continued shaking and cooling.

It is advisable to wear goggles, since the reaction is quite vigorous and chlorosulfonic acid will destroy tissue instantly. The precipitate, which is harmless, is filtered with anhydrous precautions. The reagent is then washed twice with small amounts of pyridine to remove the last traces of chloroform. It is not necessary to remove the pyridine absorbed into the salt. The reagent may then be placed in a wide mouthed bottle which should be kept tightly corked when not in use. This reagent has been observed to be stable for at least 4 months. Calculated for $(C_5H_5N)_2 \cdot SO_3$, S 13.4 per cent; found 13.5 per cent.

Benzene. c.p. benzene was used.

Petroleum ether. Dried redistilled petroleum ether, b.p. 35–60°, was used.

Extracting mixture. Redistilled absolute alcohol (free of acids) 3 parts, anhydrous ether 1 part. This was used in the Man-Gildea (4) modification of the Bloor (5) extraction method.

Acetic anhydride-pyridine mixture. Equal volumes of pyridine and acetic anhydride are mixed. This mixture is made up each time before use.

Cholesterol Standard 1. 40 mg. of cholesterol, dried in a desiccator over sulfuric acid, are dissolved in chloroform and made up to 100 cc. with this solvent. 5 cc. = 2 mg. of cholesterol.

Cholesterol Standard 2. 4 mg. of cholesterol are dissolved in 100 cc. of chloroform. 5 cc. = 0.2 mg. of cholesterol.

Cholesterol Standard 3. 20 mg. of cholesterol are dissolved in 100 cc. of chloroform. 2 cc. = 0.4 mg. of cholesterol.

Cholesterol Standard 4. 2 mg. of cholesterol are dissolved in 100 cc. of chloroform. 2 cc. = 0.04 mg. of cholesterol.

Extraction of Lipids from Serum—2 cc. of serum are extracted, according to the technique described by Kirk, Page, and Van Slyke (6) for plasma. When the amount of blood serum is limited, 0.2 cc. of serum may be used, in which case the extracting fluid is proportionately reduced. For the recovery experiments, added cholesterol and cholesteryl acetate are introduced in alcoholic solution before refluxing the mixture of serum and alcohol-ether.

Determination of Free Cholesterol—The extract, representing 2 cc. of serum, is evaporated at 60° according to the Kirk, Page, and Van Slyke (6) technique. The residue is taken up in 1 cc. of

benzene. An aliquot, 0.5 cc., of this solution is transferred to a 15 cc. centrifuge tube to avoid the necessity for continued washing during the transfer. For smaller quantities of serum, all of the extracted cholesterol is used for the determination instead of a 50 per cent aliquot.

The extract of 0.2 cc. of serum is evaporated at 60° as above. However, the residue here is extracted three times with 1 cc. of benzene. The benzene extracts are then transferred to a 15 cc. centrifuge tube and the combined extract is evaporated to about 0.5 cc. This is accomplished by heating the tube at 60° and passing a stream of air above the surface of the liquid in the tube.

To either of the above benzene extracts, 0.1 cc. of the acetic anhydride-pyridine mixture is added and the solution is well shaken. To this mixture about 10 to 20 mg. (a knife tipful) of dry pyridine sulfate are added and the tube is again well shaken. The added reagent is stirred and crushed with a stirring rod fitted with a 1-hole rubber stopper which is left in the tube during the subsequent procedure as an aid in stirring and keeping out moisture. The mixture is then heated at 45–47° in an electric oven. After 10 to 15 minutes the tube is removed, the contents stirred again, and the tube replaced for 10 minutes more. It is then transferred to an ice box, where it is allowed to remain for about 20 minutes. 5 cc. of cold petroleum ether are added, with vigorous shaking and stirring. The tube is then replaced in the ice box, where it is allowed to remain for 40 minutes. The tube is then centrifuged at 2000 to 3000 R.P.M. for 10 minutes. The supernatant fluid is decanted or aspirated. The precipitate is then washed with two 4 cc. portions of cold petroleum ether, with stirring and centrifuging in each case as before. The combined washings are reserved for the determination of the cholesterol esters as described below. The last traces of petroleum ether are removed from the precipitate in the aforementioned electric oven at 45°. 5 cc. of chloroform are added, followed by 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid, and the mixture is stirred and allowed to stand for 10 minutes in the dark. The color produced is compared in a colorimeter with the color produced on 5 cc. of the cholesterol Standard 2 similarly treated.

In the microprocedure (0.2 cc. of serum) the color is developed

by adding 2 cc. of chloroform, 1 cc. of acetic anhydride, and 0.04 cc. of sulfuric acid. This is compared to the color produced by 2 cc. of Standard 4, similarly treated.

Determination of Cholesterol Esters—The petroleum ether washings are evaporated to dryness in a 50 cc. beaker or extraction flask. The residue is hydrolyzed with alcoholic KOH after the method of Kirk, Page, and Van Slyke (6). The solution is then neutralized to phenol red by 0.1 N HCl. The mixture is evaporated to dryness, extracted with benzene, and the procedure above followed for the determination of free cholesterol. For the determination on 1 cc. of serum comparisons are made against Standard 1. For 0.2 cc. of serum, comparisons are made against Standard 3.

The cholesterol esters may also be determined by subtracting free cholesterol from the total cholesterol value in two independent determinations when sufficient serum is available and time is a factor.

Determination of Total Cholesterol—The total cholesterol may be taken as the sum of the free cholesterol and the cholesterol esters as determined above.

A direct determination may be made by saponifying the serum directly with alcoholic alkali and then evaporating to dryness. The cholesterol may then be extracted and determined as above for free cholesterol. Standards 1 and 3 are used respectively for 2 cc. and 0.2 cc. portions of serum.

Discussion of Procedure

Careful attention should be given to the details of the technique. The stirring must be vigorous and thorough at all times, especially after the addition of the pyridine sulfate reagent to insure the complete conversion of the cholesterol to the pyridine sulfate derivative.

The optimum temperature of precipitation must be carefully observed to complete the reaction in the given time. With lower temperature a more prolonged period is required, while at higher temperatures the reaction is not completed.

The optimum temperature for completion of the reaction varies with the relative amounts of pyridine and acetic anhydride; this is illustrated in Table I.

TABLE I

Study of Optimum Conditions for Isolation of Cholesterol As the Pyridine Cholesteryl Sulfate

Ratio of pyridine to acetic anhydride	Temperature	Recovery
	^{°C.}	per cent
1:0*	60	60
1:1	40	60
	46	100
	50	95
	55	90
	60	85
	80	85
	80	85
5:4	40	60
	46	95
	50	96
	55	96
	60	96
	80	90
	80	90
6:4	40	60
	46	82
	50	85
	60	94
	80	90
	40	60
	46	80
7:3	50	82
	60	90
	65	94
	70	92
	80	92
	40	68
	46	98
4:5	50	92
	60	80
	80	75
	40	70
	46	90
	52	88
	60	80
4:6	80	70

With pure acetic anhydride plus traces of pyridine adsorbed to the reagent (ratio of pyridine to acetic anhydride 0:1), results were not consistent. At times good recoveries were made; at times the recoveries were very poor.

*Changes from room temperature to 80° did not improve the results.

It is apparent from Table I that the lowest optimum temperature is reached when the relative amounts of pyridine and acetic anhydride are 1:1. Other concentrations do not give as complete precipitation, as shown by the fact that the supernatant liquid contained some cholesterol.

For less accurate work, such as would be required in routine clinical procedure, only the free cholesterol need be determined by this method. The combined cholesterol or the total cholesterol may be determined by the usual Bloor colorimetric procedure. This, of course, will give somewhat higher values, owing to chromogenic substances other than cholesterol.

TABLE II

Recovery (in Mg.) of Free Cholesterol Added to Lipid Extract of Normal Serum

Free cholesterol (0.029 mg. present)	Sample No.							
	1	2	3	4	5	6	7	8
Added.....	0.025	0 05	0.100	0 200	0 400	0 600	1 000	1.400
Calculated.....	0.054	0 079	0 129	0 229	0 429	0 629	1 029	1 429
Found.....	0 056	0 080	0.127	0 227	0 430	0 627	1 033	1 425

TABLE III

Recovery (in Mg.) of Cholesteryl Acetate Added to Lipid Extract of Normal Serum

Esterified cholesterol	Sample No.						
	1	2	3	4	5	6	7
Present.....	0.280	0.290	0.285	0 289	0 288	0.288	0.287
Added.....	0.025	0.050	0 100	0 200	0.400	0.600	1.000
Calculated.....	0.305	0.340	0.385	0 489	0.688	0.888	1.287
Found.....	0.300	0.342	0.379	0 479	0 678	0.872	1.200

Results

Representative results of the recovery of various amounts of cholesterol in benzene which were precipitated according to the procedure are shown below.

Sample No.....	1	2	3	4	5	6	7
Present, mg.....	0.025	0.05	0.10	0.20	0.40	0.80	1.00
Found, mg.....	0.024	0.049	0.099	0.199	0.398	0.795	0.99
Recovered, %.....	96	98	99	99	99	99	99

It can be seen that quantities as small as 0.025 mg. were recovered within 4 per cent error. The completeness of precipitation was checked by examining the petroleum ether washings for cholesterol. The washings were free of cholesterol in all cases, or the color, if any, was so faint that it was not observable. The errors are partly due to the error in reading the color.

Representative results of the recovery of added free cholesterol to the lipid extract of normal sera are given in Table II from which it can be seen that the recovery of added cholesterol is within the experimental error of the method. Added cholesterol acetate had no effect upon these determinations.

In another series of experiments cholesterol acetate was added to the serum extract, the free cholesterol was determined, and then the esters were hydrolyzed and determined as pyridine cholesteryl sulfate. The results of these experiments are shown in Table III, from which it can be seen that the recovery of added cholesterol esters is also quantitative. Thus, both esterified and free cholesterol can be recovered quantitatively from the lipid extract. Hence, the total cholesterol may also be determined accurately by this new procedure.

SUMMARY

1. A simple and accurate method is described for the separation and determination of small amounts of cholesterol in the presence of cholesterol esters and other lipids.

2. This separation is effected by the conversion of cholesterol to pyridine cholesteryl sulfate and extraction of the unchanged esters and lipids with petroleum ether.

3. The advantages of the conversion to this derivative over the conversion to the digitonide are discussed.

4. Added cholesterol to the lipid extracts of sera may be quantitatively recovered, while added cholesteryl acetate may be recovered in the washings, since it does not react with the reagent.

5. Added cholesteryl acetate may be quantitatively determined after hydrolysis.

6. Free, ester, and total cholesterol may be determined on the same sample.

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FRACTIONATION OF CHOLESTEROL IN BLOOD BY PRECIPITATION AS PYRIDINE CHOLESTERYL SULFATE AND CHOLESTEROL DIGITONIDE*

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In the accompanying paper (1), the authors reported a new method for the determination of free cholesterol in blood serum as the pyridine cholesteryl sulfate. This method as applied to blood was withheld for more than a year because of the unusually low values obtained for free cholesterol in normal sera as compared to the values obtained by precipitation with digitonin. The values obtained by this method were about one-third of those obtained by the digitonin method, 6 to 10 per cent of the total cholesterol being precipitated by this method, while 25 to 35 per cent of the total cholesterol was precipitated by digitonin. The latter has been the hitherto accepted value for free cholesterol in normal sera.

Table I shows the difference in the values obtained by the two methods. Three different methods of extraction were used (2-4) on each serum to eliminate the mode of extraction as a variable factor which might account for any differences.

At first, it was held possible that the precipitation might not be complete. However, quantitative recoveries of added cholesterol on numerous samples, even in the presence of added cholesteryl acetate, checked the validity of the method (1). The question that some form of free cholesterol may not be determined by this method was next investigated. In Table II the total cholesterol values are compared, first by a colorimetric determination of the

* Presented before the American Society of Biological Chemists at Washington, March 25-28, 1936 (*Proc. Am. Soc. Biol. Chem.*, **8**, xxviii; *J. Biol. Chem.*, **114** (1936)).

whole lipid extract and then after saponification as the digitonide and as the pyridine cholesteryl sulfate. It may be observed from Table II that the total cholesterol values by the new method are even slightly higher than those obtained by the digitonin method. Thus, additional evidence is offered to show that when the hydroxyl group of cholesterol is free, it will be completely precipitated by the new method. The higher values of the direct colori-

TABLE I

Free Cholesterol Values (in Mg. per 100 Cc. of Serum) by Pyridine Cholesteryl Sulfate and Cholesterol Digitonide Methods

Specimen No.	Kirk <i>et al.</i> (2)		Bloor (3)		Dry method (4)	
	Pyridine cholesteryl sulfate	Cholesterol digitonide	Pyridine cholesteryl sulfate	Cholesterol digitonide	Pyridine cholesteryl sulfate	Cholesterol digitonide
1	16	53	16	52	17	55
2	21	66	20	65	20	66
3	21	57	20	57	21	56
4	23	65	24	64	24	66
5	12	68	13	65	12	68
6	18	70	17	70	18	72

TABLE II

Comparative Estimations of Total Cholesterol (in Mg. per 100 Cc. of Serum) by Various Methods

Specimen No.....	1	2	3	4	5	6	7	8	9	10
Pyridine cholesteryl sulfate.....	204	154	211	200	234	209	215	158	268	217
Cholesterol digitonide.....	190	147	200	190	220	195	207	154	250	209
Direct colorimetric.....	222	176	228	215	252	220	204	170	287	231

metric determination are due partly to the presence in serum of chromogenic compounds other than cholesterol.

Before any conclusions are drawn as to the significance of the difference between the free cholesterol values by the two methods, a comparison of the chemistry of the two procedures should be made. The formation of cholesteryl pyridine sulfate depends upon a well understood reaction of the hydroxyl group of cholesterol (5-8). On the other hand, the formation of cholesterol digitonide is not well understood, partly because the structure of

digitonin is still uncertain. In addition, there is no certainty that cholesterol must be free before it can react with digitonin. Moreover, although it is well known that digitonin will not precipitate fatty acid esters of cholesterol, there is still a possibility that a loosely bound cholesterol derivative might be split by this reagent. In a recent review on the subject Bills (9) points out the great many combinations that cholesterol is known to enter into. It is possible that a complicated mixture like blood serum may contain cholesterol in combinations other than that of the fatty acid ester type. Consequently, to explain the higher values obtained for free cholesterol by the digitonin method, one may postulate that in addition to free cholesterol loosely bound cholesterol, not of the fatty acid ester type, is precipitated. This might be accomplished

TABLE III
Fractionation of Cholesterol (in Mg. per 100 Cc.) in Normal Sera

Cholesterol	Specimen No.									
	1	2	3	4	5	6	7	8	9	10
Unbound.....	18	14	21	19	20	17	19	13	21	21
Loosely bound.....	42	38	50	43	54	33	39	37	58	49
Free	60	52	71	62	74	50	58	50	79	70
Ester	144	102	140	138	160	159	157	108	189	147
Total.....	204	154	211	200	234	209	215	158	268	217

in two ways, either by splitting the loose combinations first or by precipitating the whole complex.

Precipitation as the pyridine cholesteryl sulfate then becomes a valuable addition to the understanding of the distribution of cholesterol in the blood serum in health and disease. The cholesterol in the blood can now be divided into three groups: first, the cholesterol determined by precipitation as the sulfate salt; second, the cholesterol precipitated as the digitonide but not as the sulfate; third, the cholesterol neither precipitated as the digitonide nor as the sulfate salt unless saponified. That cholesterol must exist in the blood serum in more fractions than just "ester" and "free" cholesterol has been previously pointed out (4). Table III shows the fractionation of cholesterol in the blood serum of normal adults. "Unbound cholesterol" is that precipitated as

the pyridine cholesteryl sulfate. "Free cholesterol" is the fraction precipitated by digitonin. "Loosely bound cholesterol" is the difference between the free cholesterol and unbound cholesterol. "Ester cholesterol" is the difference between total cholesterol and free cholesterol. "Total cholesterol" was determined as the pyridine cholesteryl sulfate after saponification. These total cholesterol values may be compared in Table II to the total cholesterol values obtained by the other two methods.

The values in Table III are representative of a series of twenty-five normal adults. It may be observed that the ratio of free to total cholesterol has the constancy reported recently by Sperry

TABLE IV

Fractionation of Cholesterol in Citrated Red Blood Cells

The values are expressed as mg. of cholesterol.

Specimen No.....	1	2	3	4
Unbound cholesterol....	0.56	0.68	0.85	0.90
Free cholesterol.....	0.59	0.71	0.80	0.94
Cholesterol in washings of pyridine cholesteryl sulfate ppt.*.....	0.05	0.06	0.07	0.06
Cholesterol in washings of cholesterol digitonide ppt.†.....	0.03	0.03	0.03	0.03

* Determinations were made by a direct colorimetric estimation. The duplicate washings were combined in view of the small quantities involved.

† The washings of the precipitate were taken to dryness and then extracted with ether to eliminate digitonin. Here again the combined washings of the duplicates were used in a direct colorimetric estimation.

(10). The terms "unbound" and "loosely bound" cholesterol are chosen temporarily in the absence of better terms to denote the two fractions into which the digitonin-precipitable "free cholesterol" may be divided by the new method. Variations of these fractions in disease will be of interest and it is hoped that this will result in a more thorough knowledge of the significance of cholesterol in blood serum.

In view of the wide difference between the unbound cholesterol and free cholesterol values in serum, it seemed to be of great interest to compare the two values in red cells, especially since free cholesterol comprises almost all the cholesterol in the cells (11, 12).

Determinations were therefore made on red cells obtained from citrated blood in which the red cells were repeatedly washed with physiological saline to remove all the serum. Representative values of these results are given in Table IV.

It is seen in Table IV that the difference between unbound and free cholesterol is negligible. Thus the fraction which was named by us "loosely bound cholesterol" is practically absent in citrated red cells. The relative amount of ester cholesterol is small, confirming the reports in the literature (11, 12). The ester cholesterol was determined directly, without any preliminary saponification and isolation, and therefore the values may be slightly high owing to other chromogenic substances.

TABLE V

Fractionation of Cholesterol in Red Blood Cell Clot

The values are expressed as mg. of cholesterol.

Specimen No.....	1	2	3	4	5	6
Unbound cholesterol.....	1.53	1.32	1.20	0.96	1.46	1.36
Free cholesterol.....	1.71	1.50	1.50	1.15	1.50	1.43
Cholesterol in washings of pyridine cholesteryl sulfate ppt.*...	0.75	0.60	0.62	0.32	0.29	0.30
Cholesterol in washings of cholesterol digitonide ppt.*.....	0.60	0.40	0.30	0.20	0.22	0.25

* See Table IV.

In view of the almost complete absence of the loosely bound cholesterol in the citrated red cells it appeared desirable to eliminate the possible effect of the anticoagulant. The cholesterol fractions were therefore studied on the red cell clot rinsed with saline. Naturally, such clots contained some occluded serum. The results are presented in Table V.

It may be observed in Table V that here again the difference between unbound and free cholesterol is small, although somewhat larger than in the case of washed red cells. The ester cholesterol values are also relatively higher. This difference is no doubt due to the presence of some occluded serum. The general trend of the distribution is that which one would expect if a small amount of serum were added to the washed red cells. It appears therefore

that sodium citrate has no appreciable effect upon the cholesterol fractions in the red cells.

It may be concluded therefore that most of the cholesterol is unbound in the red cell, while in normal serum a relatively small percentage of the cholesterol is unbound. This may be of significance in that cholesterol is known to act so as to protect the red cells from hemolysis by foreign substances such as saponin and the bile acids (9). Practically all of the loosely bound and most of the ester cholesterol are in the serum. It is hoped that a clew to the fundamental physiological significance of these findings may be found in an extensive study in disease and various experimentally induced conditions.

Procedure

The blood sera used in these experiments were obtained from apparently normal cases and extracted by three different methods as described below. The citrated red cells were obtained by adding 5 cc. of whole blood to 15 cc. of a 0.5 per cent sodium citrate solution in 0.9 per cent saline. This mixture was centrifuged and washed three times with 15 cc. of 0.9 per cent saline in a graduated centrifuge tube. The residue was extracted by refluxing with an alcohol-ether mixture according to the technique of Kirk *et al.* (2). Red cell clots were obtained by drawing blood without an anti-coagulant and then letting it stand for 2 hours in an ice box. The serum was separated by centrifuging and the clot rinsed twice with 0.9 per cent saline. The whole clot obtained in this manner was extracted as in the case of the citrated red cells.

Extraction Methods

Kirk, Page, and Van Slyke (2)—2 cc. of serum were extracted and the lipids prepared according to the above authors. The petroleum ether solution of the lipids was taken to dryness at 60° in a small flask and taken up in exactly 2 cc. of benzene. When well stirred, the lipids are easily soluble in cold benzene. 0.5 cc. aliquots are then used for the free cholesterol by the pyridine cholesteryl sulfate method and the digitonin method. Another 0.5 cc. portion was transferred to a test-tube for saponification. The remaining 0.5 cc. may be used for the regular Liebermann-Burchard reaction.

Wet Extraction by Bloor's Method (3)—2 cc. of serum were treated with 50 cc. of alcohol-ether mixture and heated to boiling. The mixture was filtered and the precipitate washed with the 25 cc. portions of alcohol-ether heated to boiling. The extract was dried on a water bath. The lipids were then taken up in petroleum ether and filtered. The technique from this point was carried on as above, the same pipettes being used for the benzene. It was later found that the extraction step with petroleum ether could be omitted. The lipids after the alcohol ether was removed could be taken up directly in benzene.

Dry Extraction (4)—2 cc. of serum were dried on paper and extracted for 1 hour with 3 per cent pyridine in alcohol according to the technique described previously (4). The extract, after removal of the pyridine-alcohol solvent *in vacuo*, was taken up in petroleum ether, filtered, and treated the same as the others. Here again the lipids may be taken up directly in benzene, the petroleum ether extraction being omitted.

Estimation of Unbound Cholesterol—The procedure used was described in our previous paper (1) for the estimation of free cholesterol as the pyridine cholesteryl sulfate. This fraction is referred to as unbound cholesterol in this paper, in order to prevent confusion with the values obtained by the digitonin method.

Estimation of Free Cholesterol—The procedure used is a modification of the technique described by Schoenheimer and Sperry (13), so as to allow the use of larger quantities of serum and so that application could be made to the lipid extracts regardless of method of extraction. 0.5 cc. portions of the benzene extract equivalent to 0.5 cc. of serum were transferred to a 15 cc. graduated centrifuge tube and the benzene removed by heating to 60° and passing an air current over the surface. The lipids were then taken up in 5 cc. of warm 95 per cent alcohol and warmed at 60° until dissolved. The mixture was allowed to cool and the volume adjusted to 5 cc. 0.5 cc. of a 1 per cent digitonin solution in 50 per cent alcohol and 0.75 cc. of water were added. The mixture was well shaken and then allowed to stand for 24 hours at room temperature. The precipitate was sedimented by centrifuging for 10 minutes and washed twice with 4 cc. portions of acid-free ether in the same manner. The precipitate during the washing was well stirred with a stirring rod which is reserved for the same tube.

1 cc. of acetic acid was added and the precipitate well broken up with the stirring rod. The tube was then placed in a water bath and warmed until solution was obtained. The mixture was cooled to room temperature, 2 cc. of acetic anhydride and 0.1 cc. of sulfuric acid were added, and the mixture well stirred and placed in the dark for 27 minutes. It was then compared in a small cup colorimeter against a standard cholesterol solution in alcohol treated in the same manner as above. The amount of cholesterol in the standard was 0.4 mg. In test experiments on pure cholesterol about 97 per cent of the original amount was recovered. Recoveries of cholesterol added to the lipid extract were within 95 per cent.

Estimation of Total Cholesterol—The 0.5 cc. portions obtained from the various extractions were freed of benzene and heated to 85° with 5 cc. of saturated alcoholic potassium hydroxide. This was followed by the procedure described by Kirk *et al.* (2). As the extraction of the unsaponifiable fraction is rather irksome, the following modification was found to give good results. The saponified material was carefully neutralized with alcoholic hydrochloric acid (with methyl red indicator). 25 cc. of ether were added and thoroughly mixed. The mixture was allowed to stand for 15 minutes, filtered, and washed with ether. The ether extract was evaporated and taken up in benzene. Aliquot portions of this extract were used to determine the total cholesterol by the cholesterol digitonide and the pyridine cholesteryl sulfate methods. The technique employed for the digitonide procedure is the same as that described above under free cholesterol, while the technique for total cholesterol by the pyridine cholesteryl sulfate method was described earlier by the authors (1). For the estimation of total cholesterol by a direct colorimetric procedure the Liebermann-Burchard colorimetric reaction was applied directly to the extract from an aliquot of the lipid extract and compared against a suitable standard. In our hands the pyridine cholesteryl sulfate method was far superior to the digitonin method for the determination of total cholesterol in the red cells after saponification.

SUMMARY

1. A comparative study was made of the values obtained in normal sera by the pyridine cholesteryl sulfate and the cholesterol digitonide methods.

2. The free cholesterol values were from 6 to 10 per cent of the total by the pyridine cholesteryl sulfate and 25 to 35 per cent of the total by the cholesterol digitonide methods.

3. The total cholesterol values obtained on the saponified serum were slightly higher by the pyridine cholesteryl sulfate than by the cholesterol digitonide procedure.

4. The higher free cholesterol values by the digitonin method may be due to the splitting of loosely bound cholesterol or the precipitation of some form of combined cholesterol other than the fatty acid ester type.

5. The terms unbound cholesterol and loosely bound cholesterol are introduced to denote the two fractions into which the so called free cholesterol is divided in this investigation.

6. In red blood cells most of the cholesterol is unbound.

7. The cholesterol fractions in sera were not influenced by the three different methods of extraction employed. The sodium citrate used as an anticoagulant had no apparent effect upon the cholesterol fractions in the red cells.

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CONFIGURATIONAL RELATIONSHIP OF MEMBERS OF DISUBSTITUTED ACETIC AND PROPIONIC ACIDS CONTAINING AN ETHYL GROUP

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WITH THE ASSISTANCE OF MARTIN KUNA

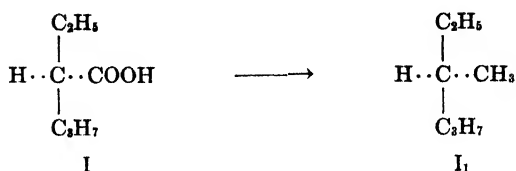
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The present investigation had a fourfold aim: (1) to establish the direction of rotation of the individual members of the homologous series of disubstituted acetic acids containing an ethyl group; (2) to correlate the configurations of the members of the homologous series of disubstituted acetic acids containing an ethyl group with those containing a methyl group; (3) to test the previously reported conclusions regarding the direction of rotation of 2-ethylvaleric acid (1) and of the higher members of the same homologous series; (4) to correlate the configurations of the members of the homologous series of disubstituted propionic acids containing an ethyl group with those containing a methyl group.

1. The rotations of two consecutive members of the homologous series of disubstituted acids were established on the basis of the direction of rotation of the hydrocarbons derived from the acids by converting the carboxylic groups into methyl groups. It is evident that two configurationally related acids (I) and (II) should yield two configurationally related hydrocarbons (I_1) and (II_1).

Inasmuch as the two hydrocarbons (I_1) and (II_1) are both dextrorotatory, it follows that those two acids which form dextrorotatory hydrocarbons will be configurationally related. It was found that such acids were both dextrorotatory. The intermediate steps leading to the hydrocarbons are given in Table I.



Dextro

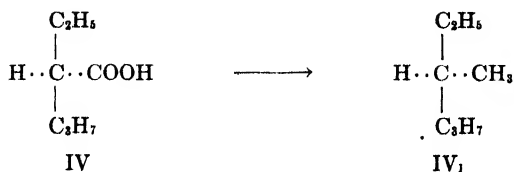


Dextro

2. This problem likewise can be solved by comparing the direction of rotation of the hydrocarbons derived from two corresponding acids. From formulæ (III) and (III₁) on the one hand, and (IV) and (IV₁), on the other, it can be seen that configurationally related 2-methylpentanoic acid (1) and 2-ethylpentanoic acid (1) should yield hydrocarbons of opposite sign.



Levo



Dextro

It was found that acids (III) and (IV) were both dextrorotatory, thus showing that these two members of the two configurationally related homologous series rotate in the same direction.

Incidentally, it may be added that since the maximum rotations of the hydrocarbons are known, it is also possible to evaluate the least maximum¹ rotation of each acid containing an ethyl group.

3. In a previous communication the levorotatory 3-propylpentanoic acid (1)² was correlated to the dextrorotatory 3-butylpentanoic acid (1). Subsequent analysis of the rotatory dispersion curves revealed a normal course of dispersion in both acids. This finding was in disagreement with the previous conclusions regarding the sign of rotation of the two configurationally related acids. It therefore seemed desirable to prepare the two acids by a different procedure, starting with 1-bromo-2-ethylpentane and 1-bromo-2-ethylhexane of known configuration by the set of reactions given in Table II.

From Table II it may be seen that the two configurationally disubstituted acetic acids lead to two disubstituted propionic acids of the same sign of rotation. Hence the correlation reported in the previous communication² should be corrected to conform with the present results.

4. Inasmuch as the disubstituted acetic acids containing a methyl group have been correlated with those having an ethyl group, and inasmuch as each of these series is correlated to a corresponding series of disubstituted propionic acids, it follows that the configuration of the series of disubstituted propionic acids containing a methyl group and of the series containing an ethyl group, likewise are correlated.

From Table II it can be seen that in the ethyl series the propionic acids rotate in opposite direction from that of the acetic acids. The same phenomenon is observed in the case of the methyl series (with the exception of the lowest member). Hence it follows that disubstituted propionic acids containing an ethyl group rotate in the same direction as the corresponding acids containing a methyl group.

Analysis of Rotatory Dispersion Curves—The results of the analysis of the rotatory dispersion curves of the disubstituted acetic and propionic acids belonging to the methyl series have

¹ The least maximum molecular rotation is defined as the minimum possible value for the maximum molecular rotation.

² Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 687 (1931).

TABLE I
Rotations of Configurationally Related Disubstituted Acetic Acids and Derivatives Containing an Ethyl Group (Homogeneous)

	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_3\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOC}_2\text{H}_5 \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{OH} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{I} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_3 \\ \\ \text{C}_3\text{H}_7 \end{array}$
Observed $[\text{M}]_D^{25}$	+2.2°	+1.9°	+1.0°	+0.7°	+2.19°
Calculated $[\text{M}]_D^{25}$ maximum.	+6.5°	+7.3°	+3.9°	+3.0°	+9.9°
	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_3\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOC}_2\text{H}_5 \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{OH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{I} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_3 \\ \\ \text{C}_4\text{H}_9 \end{array}$
Observed $[\text{M}]_D^{25}$	+6.0°	+5.8°	+2.0°	+6.19°	+4.42°
Calculated $[\text{M}]_D^{25}$ maximum.	+23.5°	+22.6°	+7.7°	+24.0°	+11.4°

TABLE II
Direction of Rotation of Disubstituted Acetic and Propionic Acids Containing an Ethyl Group. $[M]_D^{25}$ Maximum

$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{Br} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{MgBr} \\ \\ \text{C}_3\text{H}_7 \end{array}$	$\xrightarrow{+\text{CO}_2}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COOH} \\ \\ \text{C}_3\text{H}_7 \end{array}$	-3.41°
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{Br} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{MgBr} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\xrightarrow{+\text{CO}_2}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	-7.41°
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{Br} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{MgBr} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\xrightarrow{+\text{CO}_2}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$+23.5^\circ$
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{Br} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{MgBr} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$\xrightarrow{+\text{CO}_2}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{COOH} \\ \\ \text{C}_4\text{H}_9 \end{array}$	$+19.4^\circ$

already been reported in a previous communication.³ The dispersion data concerning the acids of the ethyl series in heptane solution may be seen in Table III. In order to present the data in a more compact form, only the experimental α^{25} values are

TABLE III
Rotatory Dispersions of Disubstituted Acetic and Propionic Acids in Heptane

γ	I ^a α^{25}	II α^{25}	III α^{25} (homogeneous)	IV α^{25}	V α^{25}	VI α^{25}
6678.1					-0.685	
5892.6				-0.438		
5875.6	2.253	8.011	5.833		-0.915	0.799
5780.1	2.328	8.286	6.047	-0.456	-0.950	0.818
5460.7	2.626	9.386	6.833	-0.519	-1.083	0.904
4358.3	4.35	15.59 _s	11.27 _s	-0.90	-1.86 _s	1.34 _s
4046.6	5.17	18.56	13.37	-1.10		1.48
3800		2.15				0.80
3525			9.25			(λ 3680, 10 cm.)
3500		2.65				
3375		2.90				
3340	0.83					
3275			11.25			
3160			12.25			
3070	1.03		13.25			
2980			14.25			
2900		4.40	15.25			
2840			16.25			
2800		4.90				
2740	1.43		17.85			
2630		5.90				
2600	0.83 (5 cm.)					

*The roman numerals represent the acids tabulated in the text.

given and numerals head each column. The numerals stand for the following acids.

I = *dextro-2-ethylvaleric acid* (I), $d_4^{25} = 0.9098$ (in *vacuo*); $n_D^{25} = 1.4178$; concentration 0.9249 M; visible region $l = 100$ cm., ultraviolet ~ 2.100
 region $l = 10$ cm.; $[M]_{\max}^{25} \lambda^2 = 0.024$

³ Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Physic.*, **1**, 662 (1933).

- II = *dextro-2-ethylcaproic acid* (1), $d_4^{25} = 0.9031$ (in *vacuo*); $n_D^{25} = 1.4229$; concentration 0.8587 M; visible region $l = 100$ cm., ultraviolet region $l = 10$ cm.; $[M]_{\max}^{25} = \frac{\infty 8.500}{\lambda^2 - 0.0263}$
- III = *dextro-ethyl ester of 2-ethylcaproic acid* (1), $d_4^{25} = 0.8586$ (in *vacuo*); $n_D^{25} = 1.4123$; visible region $l = 20$ cm., ultraviolet region $l = 10$ cm.; $[M]_{\max}^{25} = \frac{\infty 7.320}{\lambda^2 - 0.0232}$ (homogeneous state)
- IV = *levo-3-ethylcaproic acid* (1), $n_D^{25} = 1.4283$; concentration 0.544 M; $l = 40$ cm.; $[M]_{\max}^{25} = -\frac{\infty 1.040}{\lambda^2 - 0.042}$
- V = *levo-3-ethylcaprylic acid* (1), $n_D^{25} = 1.4344$; concentration 0.836 M; $l = 40$ cm.; $\alpha_{\text{observed}}^{25} = -\frac{0.2795}{\lambda^2 - 0.040}$
- VI = *dextro-1-iodo-2-ethylpentane* (homogeneous state), $d_4^{25} = 1.3881$ (in *vacuo*); $n_D^{25} = 1.4914$; $l = 20$ cm.; $[M]_D^{25} = \frac{\infty 1.143}{\lambda^2 + 0.034}$

On account of the low rotations of most of these substances, the dispersion data covered a rather small wave-length interval, rendering analysis difficult. The dispersion curves of all compounds belonging to the ethyl series could be expressed by a single Drude term formula within experimental error. The most significant result is the fact that both 3-ethylcaproic acid (1) and 3-ethylcaprylic acid (1) have approximately the same dispersion constant, thus excluding, as already mentioned, the possibility that these two compounds are of opposite sign when configurationally related.

It should be noted that, as in the methyl series, the substituted acetic acids are less dispersive than the substituted propionic acids. In fact, the values of the dispersive constants of the acetic acid derivatives are smaller than those of the acetic acid derivatives belonging to the methyl series. It is not impossible, since the values of λ_0 are small ($\lambda_0 = \infty 1550$), that the first partial rotation of the COOH group in the substituted acetic acids is small and of opposite sign to the observed rotation.

EXPERIMENTAL

Ethylpropylacetic Acid (2-Ethylvaleric Acid (1))—The ethylpropylacetic acid was prepared by the malonic ester synthesis.

The ethylpropylmalonic acid was recrystallized until it showed a constant melting point of 117–118°. The ethylpropylacetic acid was resolved by recrystallizing its quinine salt from acetone many times. In another experiment the resolution with cinchonidine proceeded at a faster rate.

The levo acid was recovered from the less soluble cinchonidine salt, and the dextro acid from the quinine salt. B.p. 95°, $p = 8$ mm.; $n_D^{25} = 1.4178$; $d_4^{25} = 0.9098$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-1.55^\circ}{1 \times 0.910} = -1.70^\circ; [M]_D^{25} = -2.21^\circ$$

Least maximum $[M]_D^{25} = -6.53^\circ$ (homogeneous)

0.1067 gm. substance required 8.169 cc. 0.1 N NaOH. Mol wt. 130

5.110 mg. substance: 12.090 mg. CO₂ and 4.840 mg. H₂O

C₇H₁₄O₂. Calculated. C 64.56, H 10.84

130.1 Found. " 64.57, " 10.60

Ethylpropylacetic Ethyl Ester (Ethyl Ester of 2-Ethylvaleric Acid (1))—120 gm. of ethylpropylacetic acid,

$$[\alpha]_D^{25} = \frac{+1.16^\circ}{1 \times 0.910} = +1.27^\circ \text{ (homogeneous)}$$

(from the quinine salt), were added to a solution of 360 gm. of absolute ethanol and 18 gm. of concentrated sulfuric acid. The resulting solution was refluxed for 6 hours. The ester was isolated by extraction with ether. The extract was washed free of acid and then shaken with concentrated calcium chloride solution until the washings no longer increased in volume. It was then dried over sodium sulfate and the ester was distilled. B.p. 108°, $p = 115$ mm.; yield 133 gm.; $n_D^{25} = 1.4070$; $d_4^{25} = 0.8585$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+1.00^\circ}{1 \times 0.858} = +1.17^\circ; [M]_D^{25} = +1.85^\circ \text{ (homogeneous)}$$

Least maximum $[M]_D^{25} = +7.33^\circ$ (homogeneous)

3.725 mg. substance: 8.490 mg. CO₂ and 3.495 mg. H₂O

C₉H₁₈O₂. Calculated. C 68.29, H 11.47

158.1 Found. " 68.33, " 11.54

Ethylpropyl Ethanol (2-Ethylpentanol-1)—100 gm. of ethylpropylacetic ethyl ester, $[M]_D^{25} = +1.85^\circ$ (homogeneous), were re-

duced with 120 gm. of sodium metal in 800 cc. of benzene (in four lots) with alcohol. The alcohol was isolated as usual. The ether extract was shaken with concentrated calcium chloride solution and then dried over anhydrous potassium carbonate. The alcohol boiled at 107° , $p = 102$ mm. Yield 48 gm. This was purified by the usual method of making the acid phthalic ester. The solution of the alcohol, phthalic anhydride, and pyridine was refluxed for 2 hours. The phthalic ester was purified through its sodium salt, which was made with a 5 per cent solution of sodium hydroxide, and repeatedly extracted with ether. An excess of concentrated alkali was then added to the solution of the sodium salt, and the carbinol distilled over with steam. It was then extracted with ether, and the extract was dried overnight with anhydrous potassium carbonate. The carbinol was then distilled. B.p. 107° , $p = 100$ mm.; yield 40 gm.; $n_D^{25} = 1.4250$; $d_4^{25} = 0.8280$ (*in vacuo*).

$$[\alpha]_{D}^{25} = \frac{+1.394^{\circ}}{2 \times 0.828} = +0.84^{\circ}; [M]_{D}^{25} = +0.98^{\circ} \text{ (homogeneous)}$$

$$\text{Least maximum } [M]_{D}^{25} = +3.88^{\circ} \text{ (homogeneous)}$$

3.800 mg. substance: 10.090 mg. CO_2 and 4.705 mg. H_2O

$\text{C}_7\text{H}_{16}\text{O}$. Calculated. C 72.33, H 13.89

116.1 Found. " 72.40, " 13.85

Ethylpropylethyl Bromide (1-Bromo-2-Ethylpentane)—36 gm. of ethylpropyl ethanol, $[\alpha]_D^{25} = +0.83^{\circ}$ (homogeneous), were treated with 88 gm. of phosphorus tribromide at -10° . The solution was allowed to stand at room temperature for 1 hour and then was heated on the steam bath for 20 minutes. The bromide was isolated as usual. The pentane extract was dried over phosphoric anhydride. B.p. 109° , $p = 130$ mm.; yield 32 gm.; $n_D^{25} = 1.4508$; $d_4^{25} = 1.1474$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+0.67^{\circ}}{1 \times 1.147} = +0.58^{\circ}; [M]_D^{25} = +1.04^{\circ} \text{ (homogeneous)}$$

$$\text{Least maximum } [M]_D^{25} = +4.12^{\circ} \text{ (homogeneous)}$$

5.300 mg. substance: 9.115 mg. CO_2 and 4.000 mg. H_2O

$\text{C}_7\text{H}_{16}\text{Br}$. Calculated. C 46.92, H 8.45

179.0 Found. " 46.92, " 8.44

Ethylpropylethyl Iodide (1-Iodo-2-Ethylpentane)—14 gm. of ethylpropyl ethanol, $\alpha_D^{25} = -0.61^{\circ}$, were placed in a bomb tube and

cooled in a dry ice-acetone bath. Anhydrous hydrogen iodide (about 30 gm.) was then distilled into it and the tube sealed. This was allowed to stand at room temperature for 1 week, and was then heated for 1 hour at 70°. The iodide was isolated as usual. B.p. 132–133°, $p = 160$ mm.; yield 16.5 gm.; $n_D^{25} = 1.4914$; $d_4^{25} = 1.3881$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-0.799^\circ}{2 \times 1.388} = -0.29^\circ; [M]_D^{25} = -0.66^\circ \text{ (homogeneous)}$$

Least maximum $[M]_D^{25} = -2.98^\circ$ (homogeneous)

5.300 mg. substance: 7.100 mg. CO₂ and 3.080 mg. H₂O

C₇H₁₅I. Calculated. C 37.16, H 6.69

226.0 Found. " 36.53, " 6.53

Methylethylpropyl Methane—10 gm. of ethylpropylethyl iodide, $[\alpha]_D^{25} = -0.29^\circ$ (homogeneous), were reduced in methanol and 10 per cent sodium hydroxide solution in the presence of Raney's catalyst, with hydrogen at atmospheric pressure for 2 days. The hydrocarbon was isolated as usual. B.p. 92–93°, $p =$ atmospheric; yield 3 gm.

$$[\alpha]_D^{25} = \frac{-1.50^\circ}{1 \times 0.684} = -2.19^\circ; [M]_D^{25} = -2.19^\circ$$

Maximum $[M]_D^{25} = -9.9^\circ$ (homogeneous)

2.914 mg. substance: 8.980 mg. CO₂ and 4.130 mg. H₂O

C₇H₁₆. Calculated. C 83.89, H 16.11

100.1 Found. " 84.03, " 15.86

Ethylpropylpropionic Acid (3-Ethylcaproic Acid (1))—25 gm. of ethylpropylethyl bromide, $[M]_D^{25} = +1.04^\circ$ (homogeneous), were added dropwise to 5 gm. of fine magnesium turnings in 75 cc. of dry ether. This was refluxed for 10 minutes, and then cooled with an ice-alcohol bath. Carbon dioxide was passed into the mixture, and the acid was isolated as usual. B.p. 158–159°, $p = 79$ mm.; yield 10 gm.

$$[\alpha]_D^{25} = \frac{-0.55^\circ}{1 \times 0.911} = -0.60^\circ; [M]_D^{25} = -0.86^\circ \text{ (homogeneous)}$$

Least maximum $[M]_D^{25} = -3.41^\circ$ (homogeneous)

2.965 mg. substance: 7.260 mg. CO₂ and 2.960 mg. H₂O

C₈H₁₆O₂. Calculated. C 66.61, H 11.19

144.1 Found. " 66.77, " 11.17

*Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

Ethylbutylacetic Acid (2-Ethylcaproic Acid (1))—The acid was made by the malonic ester synthesis and resolved by recrystallizing the quinine salt from 50 per cent acetone. After seven crystallizations, an acid was obtained, which had the following properties. B.p. 120° , $p = 13$ mm.; $n_D^{25} = 1.4229$; $d_4^{25} = 0.9031$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-3.79^{\circ}}{1 \times 0.903} = -4.20^{\circ}; [M]_D^{25} = -6.05^{\circ} \text{ (homogeneous)}$$

“

Maximum $[M]_D^{25} = -23.5^{\circ}$ (homogeneous)

0.1046 gm. substance required 7.205 cc. 0.1 N NaOH. Mol. wt. 145

4.800 mg. substance: 11.695 mg. CO₂ and 4.810 mg. H₂O

C₈H₁₆O₂. Calculated. C 66.61, H 11.19

144.1 Found. “ 66.44, “ 11.21

The acid recovered from the mother liquors was converted into the cinchonidine salt in acetone. The salt which crystallized very slowly was recrystallized from acetone, then from 60 per cent acetone. An acid having a rotation of

$$[\alpha]_D^{25} = \frac{+5.18^{\circ}}{1 \times 0.903} = +5.74^{\circ} \text{ (homogeneous)}$$

was obtained from the crystals.

Ethylbutylacetic Ethyl Ester (Ethyl Ester of 2-Ethylcaproic Acid (1))—250 gm. of ethylbutylacetic acid, $[\alpha]_D^{25} = -4.20^{\circ}$ (homogeneous), were esterified with 1300 cc. of absolute ethanol and 50 gm. of sulfuric acid by refluxing the solution for 5 hours. The excess alcohol was distilled through a Widmer column. The residue was extracted with ether. The extract was washed with concentrated calcium chloride solution and dried over sodium sulfate. The ester distilled at 90° , $p = 28$ mm.; yield 255 gm.; $n_D^{25} = 1.4123$; $d_4^{25} = 0.8586$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-2.90^{\circ}}{1 \times 0.858} = -3.38^{\circ}; [M]_D^{25} = -5.82^{\circ} \text{ (homogeneous)}$$

Maximum $[M]_D^{25} = -22.6^{\circ}$

4.015 mg. substance: 10.295 mg. CO₂ and 4.190 mg. H₂O

C₁₀H₂₀O₂. Calculated. C 69.70, H 11.71

172.2 Found. “ 69.92, “ 11.67

Ethylbutyl Ethanol (2-Ethylhexanol-1)—240 gm. of ethylbutylacetic ethyl ester, $[\alpha]_D^{25} = -3.38^{\circ}$ (homogeneous), were reduced

with alcohol in eight portions, 30 gm. of sodium and 200 cc. of benzene being used for each. The carbinol was isolated as usual (yield 120 gm.) and purified through its acid phthalic ester. B.p. 110° , $p = 55$ mm.; $n_D^{25} = 1.4292$; $d_4^{25} = 0.8293$ (*in vacuo*).

$$[\alpha]_{587.6}^{25} = \frac{-5.085^{\circ}}{4 \times 0.829} = -1.53^{\circ}; [M]_{587.6}^{25} = -1.99^{\circ}$$

Least maximum $[M]_D^{25} = -7.72^{\circ}$ (homogeneous)

3.584 mg. substance: 9.695 mg. CO_2 and 4.460 mg. H_2O

$\text{C}_{18}\text{H}_{18}\text{O}$. Calculated. C 73.77, H 13.94

130.1 Found. " 73.78, " 13.92

Ethylbutylethyl Iodide (1-Iodo-2-Ethylhexane)—15 gm. of ethylbutyl ethanol, $[\alpha]_D^{25} = -1.53^{\circ}$ (homogeneous), were poured into a bomb tube and cooled in a dry ice-acetone bath. Then about 25 cc. of anhydrous hydrogen iodide were distilled into it. The tube was sealed and allowed to stand at room temperature for 1 week. It was then heated for 1 hour at 70° , cooled in dry ice, and opened. The excess hydrogen iodide escaped overnight. The iodide was extracted with pentane. The extract was washed with sodium bisulfite solution, dilute sodium carbonate solution, and water, and dried over phosphoric anhydride. The iodide distilled at 60° ; $p = 2$ mm.; yield 22 gm.; $n_D^{25} = 1.4897$; $d_4^{25} = 1.3398$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-3.46^{\circ}}{1 \times 1.34} = -2.58^{\circ}; [M]_D^{25} = -6.19^{\circ} \text{ (homogeneous)}$$

Least maximum $[M]_D^{25} = -24.0^{\circ}$

6.730 mg. substance: 9.890 mg. CO_2 and 4.230 mg. H_2O

$\text{C}_8\text{H}_{17}\text{I}$. Calculated. C 39.99, H 7.14

240.1 Found. " 40.07, " 7.03

Ethylbutylethyl Bromide (1-Bromo-2-Ethylhexane)—15 gm. of ethylbutyl ethanol, $[\alpha]_{587.6}^{25} = -1.53^{\circ}$, were treated with 30 gm. of phosphorus tribromide at -10° . The solution was heated 1 hour at 100° . The bromide was isolated as usual. B.p. 110 – 111° , $p = 71$ mm.; yield 10 gm.; $n_D^{25} = 1.4526$; $d_4^{25} = 1.1197$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-2.90^\circ}{1 \times 1.12} = -2.59^\circ; [M]_D^{25} = -5.00^\circ \text{ (homogeneous)}$$

$$\text{Least maximum } [M]_D^{25} = -19.4^\circ$$

6.016 mg. substance: 10.995 mg. CO₂ and 4.800 mg. H₂O

C₈H₁₇Br. Calculated. C 49.73, H 8.88

193.1 Found. " 49.83, " 8.85

Ethylbutylpropionic Acid (3-Ethylheptanoic Acid (1))—10 gm. of ethylbutylethyl bromide, $[\alpha]_D^{25} = -2.59^\circ$, were dropped into 2 gm. of magnesium turnings in 50 cc. of dry-ether. The mixture was refluxed for a few minutes, and then cooled in an ice-alcohol bath. Carbon dioxide was then passed in and the acid isolated as usual.

$$[\alpha]_D^{25} = \frac{+1.10^\circ}{1 \times 0.908} = +1.21^\circ; [M]_D^{25} = +1.91^\circ \text{ (homogeneous)}$$

$$\text{Least maximum } [M]_D^{25} = +7.41^\circ$$

4.500 mg. substance: 11.280 mg. CO₂ and 4.635 mg. H₂O

C₈H₁₈O₂. Calculated. C 68.29, H 11.47

158.1 Found. " 68.35, " 11.52

Methylethylbutyl Methane—20 gm. of ethylbutylethyl iodide, $[\alpha]_D^{25} = -2.58^\circ$ (homogeneous), were dissolved in about 50 cc. of methanol and 25 cc. of 10 per cent alkali. Raney's catalyst was added and the mixture shaken in an atmosphere of hydrogen for 16 hours. The hydrocarbon was isolated as usual and distilled. B.p. 115°, *p* = atmospheric; yield 4 gm.

$$[\alpha]_D^{25} = \frac{-2.75^\circ}{1 \times 0.710} = -3.87^\circ; [M]_D^{25} = -4.42^\circ \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = -11.4^\circ$$

3.275 mg. substance: 10.107 mg. CO₂ and 4.685 mg. H₂O

C₈H₁₈. Calculated. C 84.11, H 15.89

114.1 Found. " 84.15, " 16.00

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **92**, 456 (1931).

OPTICAL ROTATIONS OF CONFIGURATIONALLY RELATED AZIDES

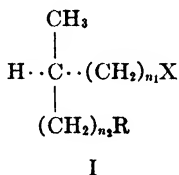
By P. A. LEVENE AND ALEXANDRE ROTHEN

WITH THE ASSISTANCE OF MARTIN KUNA

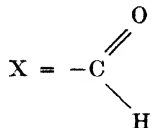
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Substances of the following type can be classified into two categories.



n_1 or $n_2 = 0$ or an integer; X = a functional group; R = a normal alkyl group, C_6H_5 or C_6H_{11} . To the first belong series of substances in which the configuration of the members having $n_1 = 0$ can be correlated by methods of classical organic chemistry to those of the members having $n_1 > 0$. The most representative series of this category is the one in whose members



To the second category belong series in which the above correlation cannot be established by the methods of classical organic chemistry. The most representative series is that in which X is a halogen.

In individual series of the first category, the following observations were made: First, the partial rotations of the functional

group of the members having $n_1 = 0$ were of opposite sign from those having $n_1 > 0$; second, a periodic change was observed in the shift of the direction of rotation of these partial contributions with the progressive increase in the value of n_1 .

In the second category of substances only the members of the series beginning with $n_1 = 1$ can be correlated by methods of classical organic chemistry and a periodic shift of rotation is observed with increase in n_1 . It was expected that by assigning alternatively a dextro- or a levorotation to the members having $n_1 = 0$, one of the series would parallel completely the events of the series of the substances of the first category. In that series the member with $n_1 = 0$ would be correlated to the members having $n_1 > 0$.

If a dextrorotation be assigned to the halides and azides having $n_1 = 0$ given in Table I, the series so obtained would resemble the series of aldehydes with respect to the periodic change in the direction of the shift of rotation with the progressive increase in the value of n_1 . On the other hand, they differ from the series of aldehydes in that no change in rotation is observed on passing from the members with $n_1 = 0$ to those with $n_1 = 1$.

If a levorotation is assigned to the members having $n_1 = 0$, series are obtained resembling those of the first category in only one respect: namely, the rotations of the members having $n_1 = 0$ are of opposite sign from those having $n_1 > 0$.

Thus, the analogy in the rotatory events observed with the progressive increase of the value of n_1 in the two categories of substances—one represented by the aliphatic aldehydes and the other by the halides—is not complete and therefore the comparison of the two sets of events fails to answer the question of the configurational relationships of substances of the second category having $n_1 = 0$ to those having $n_1 = 1$.

For the purpose of solving our problem, the azides have an advantage over the halides inasmuch as they can be converted into the corresponding amines. True, the amines, like the azides and the halides, belong to the second category of substances. However, the amines having $n_1 = 0$ can be correlated to those having $n_1 = 1$ by a sufficiently reliable argument, even though the latter is not based on methods of classical organic chemistry. The results of the conversion of the azides into the corresponding amines will be reported in the present communication and are summarized in

TABLE I
Configurationalally Related Azides, Halides, and Aliphatic Aldehydes. $[M]_D^{25}$ Maximum

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{N}_3 \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{N}_3 \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{N}_3 \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{N}_3 \\ \\ \text{C}_2\text{H}_5 \end{array}$	+17.0°
$\pm \approx 43^\circ$	+11.1°	+26.0°		
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{Br} \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{Br} \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2\text{Br} \\ \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3\text{Br} \\ \\ \text{C}_2\text{H}_5 \end{array}$	+21.9°
$\pm 30.0^\circ$	+7.9°	+38.8°		
$\begin{array}{c} \text{CH}_3 \quad \text{O} \\ \diagup \quad \diagdown \\ \text{H} \cdots \text{C} \cdots \text{C} \cdots \text{H} \\ \quad \quad \\ \text{C}_2\text{H}_5 \quad \text{H} \end{array}$	$\begin{array}{c} \text{CH}_3 \quad \text{O} \\ \diagup \quad \diagdown \\ \text{H} \cdots \text{C} \cdots \text{CH}_2 \cdots \text{C} \cdots \text{H} \\ \quad \quad \quad \quad \\ \text{C}_2\text{H}_5 \quad \text{C}_2\text{H}_5 \quad \text{H} \end{array}$	$\begin{array}{c} \text{CH}_3 \quad \text{O} \\ \diagup \quad \diagdown \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_2 \cdots \text{C} \cdots \text{H} \\ \quad \quad \quad \quad \\ \text{C}_2\text{H}_5 \quad \text{C}_2\text{H}_5 \quad \text{H} \end{array}$	$\begin{array}{c} \text{CH}_3 \quad \text{O} \\ \diagup \quad \diagdown \\ \text{H} \cdots \text{C} \cdots (\text{CH}_2)_3 \cdots \text{C} \cdots \text{H} \\ \quad \quad \quad \quad \\ \text{C}_2\text{H}_5 \quad \text{C}_2\text{H}_5 \quad \text{H} \end{array}$	+20.3°
	-8.7°	+12.0°	+12.8°	

TABLE II
Rotations of Primary Azides and Their Intermediate Derivatives

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2 - \\ \quad \quad \\ \text{C}_2\text{H}_5 \end{array}$	—OH	—I	—N ₃	—NH ₂	—CH ₂ OH	—CH ₂ I	—CH ₂ N ₃	—(CH ₂) ₂ OH	—(CH ₂) ₂ I	—(CH ₂) ₂ N ₃
$[\text{M}]_D^{25}$	—3.87°	+8.28°	+8.61°	—2.14°*	+3.33°	+16.1°	+9.63°	+3.72°	+8.20°	+5.41°†
$[\text{M}]_D^{25}$ maximum	—5.2°	+11°	+12°	—4.6°	+9.1°	+44°	+26°	+12°	+26°	+17°†
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2 - \\ \quad \quad \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$	Levo	Dextro	Levo†							
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2 - \\ \quad \quad \\ \text{C}_7\text{H}_{15} \text{ (n)} \end{array}$	—4.08°	+2.54°	—0.74°†							

* From an azide of $[\text{M}]_D^{25} = +5.42^\circ$.

† In heptane.

‡ Anomalous rotatory dispersion.

Table II. The discussion of the configurational relationship of the amines and therefore of the azides having $n_1 = 0$ to those having $n_1 = 1$ will be made the topic of a later communication. It is hoped that a comparison of the events in the series of azides with those of the series of halides will furnish a basis for the correlation of the configurations of the halides having $n_1 = 0$ with those having $n_1 > 0$.

Synthesis of the Azides—The secondary azides were prepared from the iodides by the action of sodium azide. It is worthy of note that in two azides prepared in this manner there was observed a change of sign, the rotations being of a high value.

TABLE III
Rotation of Azides and Corresponding Amines. $[M]_D^{25}$ Homogeneous

$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{N}_3 \\ \\ \text{C}_2\text{H}_5 \\ +16^\circ \end{array}$	$\xrightarrow[\text{Pt}]{\text{H}_2}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ +0.7^\circ \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{N}_3 \\ \\ \text{C}_2\text{H}_5 \\ +5.4^\circ \end{array}$	$\xrightarrow[\text{Pt}]{\text{H}_2}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{CH}_2\text{NH}_2 \\ \\ \text{C}_2\text{H}_5 \\ -2.1^\circ \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{N}_3 \\ \\ \text{C}_6\text{H}_{13} \\ +43^\circ \end{array}$	$\xrightarrow[\text{Pt}]{\text{H}_2}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{H} \cdots \text{C} \cdots \text{NH}_2 \\ \\ \text{C}_6\text{H}_{13} \\ +5.4^\circ \end{array}$			

If it be granted, on the basis of observations on substances having $n_1 > 0$, that corresponding partial rotations of the halogen atom and the azido group are of the same direction in configurationally related substances, then the conclusion will have to be reached that configurationally related secondary halides and secondary azides rotate in the same direction. It would therefore follow that the substitution of the iodine atom by an azido group, described in the experimental part, is associated with Walden inversion. On this assumption the high value of the rotation of the azido derivatives is noteworthy.

The primary azides were prepared from the corresponding alcohols of known configuration. The set of reactions leading up to them can be seen from Table II.

Synthesis of the Amines—The amines were prepared from the corresponding azides by catalytic reduction with hydrogen in the presence of Adams' catalyst (see Table III).

SUMMARY

1. A series of azides having $n_1 = 0, 1, 2$, and 3 has been prepared. The rotatory events associated with the progressive increase in value of n_1 have been compared with those observed in the series of the corresponding halides. They were found to be similar for substances having $n_1 > 0$.

2. The rotatory events observed in the series of azides and halides (classified in the same category in which members having $n_1 = 0$ cannot be correlated by methods of classical organic chemistry to those with $n_1 > 0$) were compared with those in the series of aliphatic aldehydes (the most representative series of a category of substances in which the configuration of the members having $n_1 = 0$ can be correlated to those having $n_1 > 0$ by methods of classical organic chemistry). They were found to be not similar in both categories of substances. Hence a comparison of these events cannot be used as a basis for the correlation of the members of the series of halides and azides having $n_1 = 0$ with those having $n_1 > 0$.

3. The azides were reduced to the corresponding amines. It is hoped that in the series of amines the members having $n_1 = 0$ will be correlated to those having $n_1 > 0$, thus making possible the correlation of the azides having $n_1 = 0$ with those having $n_1 > 0$.

EXPERIMENTAL

Levo-2-Iodobutane—20 gm. of butanol-2,

$$[\alpha]_D^{25} = \frac{+5.50^\circ}{1 \times 0.807} = +6.82^\circ; [M]_D^{25} = +5.05^\circ$$

were placed in a bomb tube, cooled in a dry ice-acetone bath, and 30 cc. of anhydrous hydrogen iodide were then distilled into the substance. The tube was sealed and allowed to stand at room temperature for 2 days. It was again cooled, and opened. After the excess hydrogen iodide had escaped, the substance was poured into ice and extracted with pentane. The extract was decolorized with sodium bisulfite solution, then washed free of acid

with water, and finally dried over phosphoric anhydride. The iodide was then distilled. B.p. 111–118°, $p = 1$ atmosphere; yield 38 gm.

$$[\alpha]_D^{25} = \frac{-20.80^\circ}{1 \times 1.589} = -13.1^\circ; [M]_D^{25} = -24.1^\circ \text{ (homogeneous)}$$

5.280 mg. substance: 9.355 mg. CO₂ and 4.900 mg. H₂O

C₄H₉I. Calculated. C 26.09, H 4.93

184.0 Found. " 26.32, " 4.96

Dextro-2-Azidobutane—30 gm. of 2-iodobutane, $[\alpha]_D^{25} = -13.1^\circ$ (homogeneous), were added to a solution of 13 gm. of sodium azide (Kahlbaum) in 45 cc. of water and 300 cc. of methanol. The solution was sealed in four bomb tubes and heated overnight at 80°. An equal volume of water and about 2 volumes of CaCl₂ solution were added. The oil was extracted with pentane. The extract was washed with concentrated calcium chloride solution and dried over dry calcium sulfate. The pentane was distilled off at atmospheric pressure. The azide was distilled from a boiling water bath; the pressure was regulated so that the azide just distilled. B.p. 85°, $p = 500$ mm.; yield 5 gm.; $d_4^{25} = 0.8619$ (*in vacuo*); $n_D^{25} = 1.4122$.

$$[\alpha]_D^{25} = \frac{+13.75^\circ}{1 \times 0.862} = +16.0^\circ; [M]_D^{25} = +15.9^\circ \text{ (homogeneous)}$$

3.300 mg. substance: 5.880 mg. CO₂ and 2.695 mg. H₂O

C₄H₉N₃. Calculated. C 48.44, H 9.15

99.1 Found. " 48.59, " 9.13

Dextro-2-Aminobutane—5 gm. of 2-azidobutane, $[\alpha]_D^{25} = +16.0^\circ$ (homogeneous), were dissolved in 20 cc. of methanol and 0.5 gm. of Adams' catalyst was added. This was shaken under a pressure of 3 atmospheres of hydrogen for 3½ hours. The catalyst was filtered off and hydrogen chloride in methanol was added to the filtrate. This was concentrated to dryness under reduced pressure. Benzene was added and the solution again evaporated to dryness. The crystals were taken up in ether and petroleum ether and then filtered. Yield 4 gm.

$$[\alpha]_{\text{water}}^{25} = \frac{-0.08^\circ \times 100}{2 \times 10.0} = -0.40^\circ; [M]_{\text{water}}^{25} = -0.44^\circ \text{ (in water)}$$

The solution was made alkaline with concentrated sodium methylate.

$$[\alpha]_D^{25} = \frac{+0.12^\circ \times 100}{2 \times 6.7} = +0.90^\circ; [M]_D^{25} = +0.66^\circ$$

5.100 mg. substance: 0.554 cc. N_2 at 27° and 761 mm.

$C_4H_{12}NCl$ (109.6). Calculated, N 12.79; found, N 12.37

Levo-2-Iodo-octane—30 gm. of octanol-2,

$$[\alpha]_D^{25} = \frac{+7.85^\circ}{1 \times 0.817} = +9.60^\circ \text{ (homogeneous)}$$

were placed in two bomb tubes which were cooled in a dry ice-acetone bath. Then about 20 cc. of anhydrous hydrogen iodide were distilled into each tube. The tubes were sealed and allowed to stand overnight at room temperature. They were again cooled and opened. The excess hydrogen iodide was allowed to escape overnight. The iodide was extracted with pentane. The extract was washed with a solution of potassium iodide, then with water, and dried over phosphoric anhydride. B.p. 52° , $p = 1$ mm.; yield 37 gm.; $n_D^{25} = 1.4863$; $d_4^{25} = 1.3158$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-44.0^\circ}{1 \times 1.32} = -33.3^\circ; [M]_D^{25} = -80.0^\circ \text{ (homogeneous)}$$

3.730 mg. substance: 5.495 mg. CO_2 and 2.400 mg. H_2O

$C_8H_{17}I$. Calculated. C 39.99, H 7.14

240.1 Found. " 40.17, " 7.20

Dextro-2-Azido-octane—17 gm. of 2-iodooctane, $[\alpha]_D^{25} = -33.3^\circ$ (homogeneous), were added to a solution of 8 gm. of sodium azide in 50 cc. of water and 200 cc. of methanol. The solution was heated for 7 hours at 90° in sealed bomb tubes. The procedure followed was that described for the preparation of 2-azidobutane. B.p. 68° , $p = 9$ mm.; yield 9 gm. = 81 per cent; $n_D^{25} = 1.4332$; $d_4^{25} = 0.8555$ (*in vacuo*).

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+23.9^\circ}{1 \times 0.855} = +28.0^\circ; [M]_D^{25} = +43.4^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{+3.40^\circ \times 100}{1 \times 12.4} = +27.4^\circ; [M]_D^{25} = +42.5^\circ \text{ (heptane)}$$

3.200 mg. substance: 0.755 cc. N_2 at 23° and 757 mm.

$C_8H_{17}N_3$ (155.2). Calculated, N 27.08; found, N 27.07

Dextro-2-Aminooctane—9 gm. of 2-azidooctane, $[\alpha]_D^{25} = +28.0^\circ$ (homogeneous), were dissolved in 50 cc. of methanol and 1 gm. of Adams' catalyst was added. This was shaken at a pressure of 3 atmospheres of hydrogen for $2\frac{1}{2}$ hours. The mixture was treated as described for 2-aminobutane.

A small part of the substance was converted into the chloroplatinate.

9.810 mg. substance: 0.370 cc. N_2 at 22° and 760.6 mm.

$C_{16}H_{40}N_2PtCl_6$ (668.2). Calculated, N 4.19; found, N 4.36

The hydrochloride was dried over phosphoric anhydride in a desiccator overnight.

5.470 mg. substance: 0.401 cc. N_2 at 22° and 750.5 mm.

$C_8H_{20}NCl$ (165.6). Calculated, N 8.46; found, N 8.38

$$[\alpha]_D^{25} = \frac{-0.35^\circ \times 100}{1 \times 9.0} = -3.89^\circ; [M]_D^{25} = -6.44^\circ \text{ (water)}$$

The free amine was obtained by treating the hydrochloride with concentrated KOH. The ether extract was washed with concentrated KOH, and dried over sodium. The substance was distilled. B.p. 48° , $p = 9$ mm.; yield 3.5 gm.; $n_D^{25} = 1.4220$.

$$[\alpha]_D^{25} = \frac{+3.233^\circ}{1 \times 0.7713} = +4.19^\circ; [M]_D^{25} = +5.41^\circ \text{ (homogeneous)}$$

Dextro-1-Iodo-2-Methylbutane—60 gm. of 2-methylbutanol-1, $[\alpha]_D^{25} = -4.40^\circ$ (homogeneous), were divided into three approximately equal parts and each transferred into a bomb tube, cooled in dry ice-acetone, and about 30 cc. of anhydrous hydrogen iodide were distilled into each. The sealed tubes were allowed to stand at room temperature for 1 week. They were then heated at 70° for 1 hour. The tubes were again cooled and opened. The iodide was isolated as usual. The pentane extract was dried over phosphoric anhydride. The iodide was distilled. B.p. $145\text{--}146^\circ$, $p = 1$ atmosphere; yield 110 gm.; $n_D^{25} = 1.4950$.

$$[\alpha]_D^{25} = \frac{+6.33^\circ}{1 \times 1.514} = +4.18^\circ; [M]_D^{25} = +8.28^\circ$$

Maximum $[M]_D^{25} = +11.1^\circ$ (homogeneous)

3.850 mg. substance: 4.290 mg. CO_2 and 1.930 mg. H_2O

$\text{C}_6\text{H}_{11}\text{I}$. Calculated. C 30.30, H 5.60

198.0 Found. " 30.39, " 5.60

Dextro-1-Azido-2-Methylbutane—40 gm. of 1-iodo-2-methylbutane, $[\alpha]_D^{25} = +4.18^\circ$ (homogeneous), were added to a solution of 17 gm. of active sodium azide¹ in 45 cc. of water and 350 cc. of methanol. The solution was sealed in bomb tubes and heated for $5\frac{1}{2}$ hours at 90° . The azide was isolated as described above.

The main fraction distilled at 72° ; $p = 138$ mm.; yield 13.5 gm.; $n_D^{25} = 1.4240$; $d_4^{25} = 0.8770$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+6.67^\circ}{1 \times 0.877} = +7.61^\circ; [M]_D^{25} = +8.61^\circ$$

Maximum $[M]_D^{25} = +11.6^\circ$ (homogeneous)

3.510 mg. substance: 1.137 cc. N_2 at 24° and 751 mm.

4.926 " " 9.640 mg. CO_2 and 4.220 mg. H_2O

$\text{C}_6\text{H}_{11}\text{N}_3$. Calculated. C 53.05, H 9.80, N 37.15

113.1 Found. " 53.36, " 9.49, " 36.80

Levo-1-Amino-2-Methylbutane—10 gm. of 1-azido-2-methylbutane, $[\alpha]_D^{25} = +7.60^\circ$ (homogeneous), were reduced with Adams' catalyst in methanol. The mixture was shaken for $2\frac{1}{2}$ hours in hydrogen at a pressure of 3 atmospheres. The substance was isolated as described for 2-aminobutane. The crystals were taken up in pentane and filtered. Yield 5 gm.

$$[\alpha]_D^{25} = \frac{-0.165^\circ \times 100}{10 \times 13.35} = -0.124^\circ; [M]_D^{25} = -0.153^\circ$$

Maximum $[M]_D^{25} = -0.21^\circ$ (hydrochloride in water)

4.600 mg. substance: 0.458 cc. N_2 at 26° and 751 mm.

$\text{C}_6\text{H}_{11}\text{NCl}$ (123.6). Calculated, N 11.34; found, N 11.23

An azide of $[\alpha]_D^{25} = +4.79^\circ$ (homogeneous) was reduced as above, and the free amine isolated. B.p. $40\text{--}45^\circ$, $p = 12$ mm.

$$[\alpha]_{\text{D}875.6}^{25} = \frac{+0.122^\circ}{0.5 \times 0.7505} = -2.46^\circ; [M]_{\text{D}875.6}^{25} = -2.14^\circ$$

Maximum $[M]_{\text{D}875.6}^{25} = -4.58^\circ$ (homogeneous)

¹ Newman, M. S., *J. Am. Chem. Soc.*, **57**, 733 (1935).

Levo-1-Azido-2-Methylhexane—20 gm. of 1-iodo-2-methylhexane, $[\alpha]_D^{25} = +0.37^\circ$ (homogeneous, 1 dm.), were added to a solution of 7.5 gm. of sodium azide in 50 cc. of water and 200 cc. of methanol. This was refluxed for 60 hours on a steam bath. Part of the methanol was then distilled off through a fractionating column. The residue was poured into water and extracted with pentane. The extract was washed with water and concentrated calcium chloride solution, dried with powdered calcium chloride, and distilled. B. p. $59-60^\circ$, $p = 15$ mm.; yield 2 gm.

$$\alpha_D^{25} = -0.30^\circ \text{ (homogeneous, 1 dm.)}$$

3.000 mg. substance: 0.716 cc. N_2 at 23° and 949 mm.

4.670 " " : 10.400 mg. CO_2 and 4.461 mg. H_2O

$C_7H_{15}N_3$. Calculated. C 59.52, H 10.71, N 29.77

141.1 Found. " 60.73, " 11.04, " 27.10

Dextro-1-Iodo-2-Methylnonane—10 gm. of 2-methylnonanol-1,

$$[\alpha]_D^{25} = \frac{-2.15^\circ}{1 \times 0.833} = -2.58^\circ \text{ (homogeneous)}$$

were placed in a Pyrex pressure bottle, cooled in a dry ice-acetone bath, and anhydrous hydrogen iodide distilled into it. The stopper was clamped on, and the bottle was allowed to stand at room temperature for 2 days. Then more anhydrous-hydrogen iodide was distilled into it, and the solution was allowed to stand for 2 days. The total hydrogen iodide used was 16 gm. The reaction product was poured into water and extracted with pentane. The extract was washed with water, sodium bisulfite solution, water, concentrated calcium chloride solution, and then dried over anhydrous calcium chloride. The solution was filtered and the product distilled. B. p. 86° , $p = 4$ mm.; yield 11 gm.; $d_4^{25} = 1.254$ (in vacuo).

$$[\alpha]_D^{25} = \frac{+0.85^\circ}{1 \times 1.254} = +0.68^\circ; [M]_D^{25} = +2.54^\circ \text{ (homogeneous)}$$

4.964 mg. substance: 8.175 mg. CO_2 and 3.480 mg. H_2O

$C_{10}H_{21}I$. Calculated. C 44.76, H 7.90

268.1 Found. " 44.90, " 7.83

Levo-1-Azido-2-Methylnonane—10 gm. of 1-iodo-2-methylnonane, $[\alpha]_D^{25} = +0.68^\circ$ (homogeneous), were added to a solution of

4 gm. of sodium azide in 24 cc. of water and enough methanol was added to make the solution homogeneous (about 200 cc.). This was refluxed for 24 hours. Then it was poured into water and extracted with pentane. The extract was washed with water and concentrated calcium chloride solution, then dried over anhydrous calcium chloride. The product was then distilled. B.p. 98–102°, $p = 10$ mm.; yield 5 gm.; $d_4^{25} = 0.8658$ (*in vacuo*); $n_D^{25} = 1.4430$.

$$[\alpha]_D^{25} = \frac{-0.35^\circ}{1 \times 0.866} = -0.40^\circ; [M]_D^{25} = -0.74^\circ \text{ (homogeneous)}$$

3.785 mg. substance: 0.735 cc. N_2 at 22° and 775 mm.

3.715 “ “ : 8.930 mg. CO_2 and 3.800 mg. H_2O

$C_{10}H_{21}N_3$. Calculated. C 65.51, H 11.56, N 22.94

183.2 Found. “ 65.55, “ 11.44, “ 22.91

Levo-1-Iodo-3-Methylpentane—20 gm. of 3-methylpentanol-1,

$$[\alpha]_D^{25} = \frac{-2.63^\circ}{1 \times 0.822} = -3.20^\circ \text{ (homogeneous)}$$

were placed in two Pyrex bomb tubes, cooled in dry ice-acetone, and about 25 cc. of anhydrous hydrogen iodide were distilled into each. The tubes were then sealed and allowed to stand at room temperature for 4 days. Then they were heated in water at 65° for 1 hour and placed in a dry ice-acetone bath. The tubes were opened and allowed to stand in the cooling bath overnight. The excess HI escaped. The solution was poured into ice and potassium iodide solution and extracted with pentane. The extract was washed with 10 per cent potassium iodide solution, water, and calcium chloride solution. It was dried over anhydrous calcium chloride and filtered. Phosphoric anhydride was added and the solution was allowed to stand overnight. It was then filtered and distilled. B.p. 54°, $p = 12$ mm.; yield 20 gm.; $d_4^{25} = 1.3934$ (*in vacuo*); $n_D^{25} = 1.4866$.

$$[\alpha]_D^{25} = \frac{-10.60^\circ}{1 \times 1.393} = -7.61^\circ; [M]_D^{25} = -16.1^\circ$$

Maximum $[M]_D^{25} = -43.9^\circ$ (homogeneous)

6.300 mg. substance: 7.900 mg. CO_2 and 3.550 mg. H_2O

$\text{C}_6\text{H}_{13}\text{I}$. Calculated. C 33.96, H 6.18

212.0 Found. " 34.20, " 6.31

Levo-1-Azido-3-Methylpentane—13 gm. of 1-iodo-3-methylpentane, $[\alpha]_D^{25} = -7.61^\circ$ (homogeneous), were added to a solution of 5.2 gm. of sodium azide in 30 cc. of water and 275 cc. of methanol. This was poured into a pressure bottle and heated in an autoclave at 100° for 8 hours. The product was isolated as above. B.p. $145\text{--}148^\circ$, $p = 760$ mm.; yield 2 gm. (the greater part escaped); $n_D^{25} = 1.4300$.

$$[\alpha]_{575.6}^{25} = \frac{-3.46^\circ \times 100}{4 \times 11.41} = -7.58^\circ; [M]_D^{25} = -9.63^\circ$$

Maximum $[M]_{575.6}^{25} = -26.3^\circ$ (heptane)

3.310 mg. substance: 6.885 mg. CO_2 and 3.060 mg. H_2O

$\text{C}_6\text{H}_{13}\text{N}_3$. Calculated. C 56.64, H 10.31

127.1 Found. " 56.72, " 10.31

Dextro-1-Iodo-4-Methylhexane—30 gm. of 4-methylhexanol-1, $[\alpha]_D^{25} = +3.21^\circ$ (homogeneous) (made from Kahlbaum's active amyl alcohol), were treated with anhydrous hydrogen iodide as described above. The sealed bomb tubes were allowed to stand at room temperature for 1 week and were then heated at 70° for 1 hour. The iodide was isolated as usual. Yield 23 gm.; b.p. $74\text{--}75^\circ$, $p = 13$ mm., and $124\text{--}126^\circ$, $p = 103$ mm.; $n_D^{25} = 1.4852$; $d_4^{25} = 1.3579$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+4.93^\circ}{1 \times 1.358} = +3.63^\circ; [M]_D^{25} = +8.20^\circ$$

Maximum $[M]_D^{25} = +26.2^\circ$ (homogeneous)

4.597 mg. substance: 4.750 mg. AgI

4.830 " " : 6.595 " CO_2 and 2.900 mg. H_2O

$\text{C}_7\text{H}_{15}\text{I}$. Calculated. C 37.16, H 6.69, I 56.15

226.0 Found. " 37.23, " 6.70, " 55.85

Dextro-1-Azido-4-Methylhexane—20 gm. of 1-iodo-4-methylhexane, $[\alpha]_D^{25} = +3.63^\circ$ (homogeneous), were dissolved in a solution of 7 gm. of active sodium azide¹ (made from butyl nitrite)

in 35 cc. of water and 400 cc. of methanol. The solution was sealed in five bomb tubes and heated at 100° for 14 hours. This azide was isolated as described for the others. Yield 6 gm.; b.p. 157°, $p = 418$ mm.; $d_4^{25} = 0.8636$ (*in vacuo*); $n_D^{25} = 1.4323$.

$$[\alpha]_{5875.6}^{25} = \frac{+2.302^\circ \times 100}{4 \times 15.01} = +3.83^\circ; [M]_{5875.6}^{25} = +5.41^\circ$$

Maximum $[M]_{5875.6}^{25} = +17.3^\circ$ (heptane)

3.628 mg. substance: 7.980 mg. CO₂ and 3.470 mg. H₂O

C₇H₁₁N₃. Calculated. C 59.50, H 10.71

141.2 Found. " 59.98, " 10.70

THE DISTRIBUTION OF IRON IN CERTAIN TISSUES OF NORMAL AND ANEMIC ALBINO RATS*

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Observations of the iron content of various tissues are numerous in biochemical literature. A few typical references are cited (1-10). There does not seem to have been reported, however, any comprehensive comparison between the iron content of normal and of anemic animal tissue. The present study was carried out to make such a comparison for various tissues of white rats.

EXPERIMENTAL

Of the various procedures used for inducing nutritional anemia in rats, that of Waddell, Steenbock, Elvehjem, and Hart (11) was found most effective. As soon as they could be weaned (20 to 22 days), young rats were placed upon a Guernsey milk diet. They became highly and almost uniformly anemic within 6 to 8 weeks. The milk for this purpose was milked directly into glass bottles. Success in obtaining highly anemic rats depends upon scrupulous attention to all the recognized precautions, including frequent washing of the cages; especially is this true of glass cages which, for best results, should be cleaned daily. Both glass and galvanized iron cages were used, with approximately equal results in inducing anemia. The degree of anemia obtained is indicated in Table I, which gives the hemoglobin content in gm. per 100 ml. of blood (Newcomer's method) and the erythrocyte count in millions per c.mm. Group I contained fifteen adult rats, four males and eleven females, taken from stock. Group II contained twenty-

* The experimental data in this paper are taken from the dissertation submitted by H. F. Halenz to the Graduate School of the University of Colorado in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

three selected anemic animals, eighteen males and five females. Group III contained sixteen litter controls (animals from the same litters as those of Group II), ten males and six females, which had been fed a standard normal diet.

Representative blood samples for the tests were obtained from the blood draining into the body cavity when, upon opening the anesthetized animal, a lobe of the liver was excised. This procedure obviates certain objections (12) raised against the more commonly used method of obtaining the blood from the tip of the tail. Animals of Group I were mature animals, from 200 to 300 days old, while animals of Groups II and III were between 65 and 90 days old.

Iron determinations were made—by means of Kennedy's method (1)—on liver, heart, striated muscle, kidney, and spleen.

TABLE I

Erythrocyte Counts and Hemoglobin Values of Normal and Anemic Rats

Group	Sex	Erythrocytes	Hemoglobin
		millions per c.mm.	gm. per 100 ml.
I. Normal stock rats	M.	8.08	14.08
	F.	8.76	14.68
II. Anemic rats	M.	2.97	3.53
	F.	3.38	3.36
III. Litter controls	M.	8.32	14.93
	F.	9.02	15.02

Most workers in this field seem to have assumed that approximately blood-free tissue could be obtained by bleeding the animal freely and washing the tissue. The following method was used in an attempt to determine whether or not any of the iron found in washed tissue was due to the presence of retained blood.

Erythrocyte counts and hemoglobin determinations were made on the blood of each animal when it was killed. From these data the exact ratio of erythrocyte count to hemoglobin content of the blood for each animal was calculated. A small sample of the unwashed tissue to be examined was then thoroughly macerated in Hayem's solution and careful erythrocyte counts of several samples made. The iron content of hemoglobin is known to be very constant (0.335 per cent). From the above data the hemo-

globin content of the tissue was calculated from the hemoglobin to erythrocyte ratio. This, multiplied by 0.00335, gave the iron content of the tissue due to the presence of retained blood. This method obviates the difficulties encountered in making hemoglobin determinations of more or less colored tissue extracts. Total iron determinations of other samples of the same organ (from the same animal) were then made in triplicate. From these data the actual tissue iron could be determined.

The effect of thorough washing in distilled water of small samples of tissue was investigated, the tissue being repeatedly manipulated under water and dried between layers of filter paper. It was found that most tissues (spleen excepted) could be rapidly washed several times without losing more than a small fraction of 1 per cent in weight.

The washed tissue was then analyzed according to the procedure described above. The Hayem's solution washings showed only a few stray erythrocytes. Benzidine tests were sometimes faintly positive, but usually negative. As this test sometimes shows positive for tissue extracts, these results were not considered significant.

In a series of experiments involving liver tissue from fifteen animals, the following average results were obtained, the figures in each case being calculated per 100 gm. of tissue.

	mg.
Iron content of unwashed tissue.....	20.3
“ “ “ washed “	18.44
Loss of iron in washing (difference).....	1.86
Iron content of washings (Kennedy's method).....	1.79
“ “ “ Hb in unwashed tissue determined by differential method.....	1.82

These figures seem to indicate that fresh tissue can be washed practically free of blood without any significant loss of iron aside from that contained in the blood and without any appreciable loss of tissue weight. Spleen tissue, on account of its histological nature, cannot be successfully treated in this way. In the final experiments, only unwashed spleen tissue was used. All other tissues were treated as described above.

The results obtained in the final experiments are given in Table II. No determinations were made on the kidneys of the four males in Group I.

DISCUSSION

In normal rats, the iron content of the washed tissues examined is highest in the liver, followed by heart, kidney, and striated muscle in the order given. In anemic animals, heart precedes liver tissue. The average iron content of blood-free liver tissue of Group III (normal control) and Group II (anemic) rats was 10.33 and 4.15 mg. per 100 gm., respectively. Josephs (4) found from 2 to 7 mg. for comparable material from anemic animals; Elvehjem and Sherman (5) reported 18.4 mg. per 100 gm. of dry

TABLE II
Iron Content of Various Tissues, in Mg. of Iron per 100 Gm. of Tissue

Group	Tissue	Average iron content			
		Male	Female	Both sexes	Range
I. Normal rats	Liver	8.33	10.79	10.12	6.78- 15.71
	Muscle	3.37	3.28	3.31	2.45- 3.63
	Heart	6.65	6.88	6.82	5.08- 7.88
	Spleen	33.51	71.5	61.8	16.12-106
	Kidney		6.70		4.90- 8.21
II. Anemic rats	Liver	4.18	4.06	4.15	2.76- 5.78
	Muscle	2.92	2.65	2.87	2.17- 3.63
	Heart	4.86	4.55	4.79	3.44- 6.37
	Spleen	9.58	8.73	9.42	5.09- 14.36
	Kidney	2.99	3.14	3.02	2.29- 3.96
III. Normal controls	Liver	10.39	9.87	10.33	7.47- 12.16
	Muscle	2.85	2.80	2.83	2.27- 3.84
	Heart	6.32	6.70	6.49	5.02- 7.91
	Spleen	32.17	53.2	40.4	17.02- 76
	Kidney	5.40	5.82	5.61	3.96- 7.81

liver tissue from anemic animals. For dry, normal tissue, McFarlane (8) reported from 43 to 65 mg. of iron. Elvehjem and Peterson (9) found 13.2 mg. for normal, fresh, non-blood-free material.

More uniformity exists in the case of both heart and striated muscle. In heart muscle, average values of 6.49 and 4.79 mg. for normal and for anemic animals, respectively, were found. In the case of striated muscle, the iron content for anemic rats was 2.87 mg. per 100 gm.; for the controls it was found to be 2.83 mg. Differences in iron content between the sexes are negligible in both cases.

Washed kidney tissue yielded 5.61 mg. and 3.02 mg. per 100 gm. for normal and anemic rats, respectively. It was also found that the cortex of normal washed kidney tissue has a higher iron content than the medulla. Bogniard and Whipple (2) first noticed this when working with dogs. Both the red cell count and hemoglobin values of female rats are somewhat higher than the corresponding values for male rats. Mitchell (13) also reported this in 1932 and correlated this fact with the well known observation that the condition of pronounced anemia is not produced as readily in female rats as in males.

Unwashed spleens of normal females have an appreciably higher iron content than those of males. There is no significant difference in the case of anemic animals. Variations in iron content of normal spleens are very large, as also reported by Josephs (4) for rats, and by Bogniard and Whipple (2) and by Kennedy (1) for dogs. The average amount of iron for the control animals of both sexes found in normal spleen tissue was 40.4 mg. per 100 gm. For the anemic animals this value was decreased to 9.42 mg., or a loss of 76.7 per cent. The loss of hemoglobin in the blood of the same two groups of animals was 77.2 per cent. These figures suggest that the high spleen iron is due mostly to the blood corpuscles found in it, and not to the iron content of the tissue itself. The figures also indicate that the spleen tissue proper loses little, if any, iron as the animal becomes anemic.

SUMMARY

A simple method of obtaining approximately blood-free tissue has been described, also a "differential" method of testing the efficiency of the operation. The iron content of four kinds of blood-free (washed) tissue in thirty-three normal and twenty-six anemic albino rats has been determined. Determinations of iron in normal and anemic spleen tissues not freed from blood are also given. During the development of nutritional anemia, skeletal muscle loses little or no iron, heart muscle about one-fourth of its iron, kidney nearly half, and liver somewhat more than half.

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THE ISOLATION OF THE PRINCIPAL ESTROGENIC SUBSTANCE OF LIQUOR FOLLICULI

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Since the introduction of the vaginal smear method of detecting and assaying the follicular hormone (Allen and Doisy, 1923) many investigators have studied the occurrence and distribution of estrogenic substances. Chemists interested in the sex hormones have isolated at least six different pure compounds from the urine of pregnant women and mares but until recently the nature of the active principle of the ovary was unknown.

In their early work, Allen and Doisy used liquor folliculi aspirated from sow ovaries as the source of the follicular hormone. Extensive work on the purification of the estrus-producing component had given rather potent non-crystalline products which, however, were less active than theelin. Therefore, it was assumed by many that the activity of these preparations was due to theelin. However, in October, 1934, when we obtained a preparation having a potency exceeding that of theelin, it became clear that at least a part of the activity of the follicular fluid was due to some other substance. Promptly, we tested the effect of semicarbazide on this preparation but contrary to the effect on the potency of theelin no significant alteration in the activity was observed. These observations stimulated our interest to such a degree that we have processed almost 4 tons of sow ovaries and have worked out a procedure which permits the recovery of approximately 50 per cent of the activity of liquor folliculi extracts as the pure crystalline di- α -naphthoate of dihydrotheelin.

Several important factors have contributed materially to our success in isolating one of the active principles of the ovary. Our

earlier experience in the preparation and purification of extracts and the isolation of pure crystalline estrogenic compounds has been valuable. Furthermore, a supply of theelin for experimental work has been useful. The preparation by Schwenk and Hildebrandt (1933) and others of the reduction product of the carbonyl of theelin has been of assistance. Finally, it should be stated that we have been fortunate, for if we had encountered a new and unknown compound, it would have been necessary to extract many more tons of ovaries. Complete recovery of all of the estrogenic substance as dihydrotheelin from the 4 tons of ovaries that we have processed would have given no more than 25 mg. Since our overall recovery was less than 50 per cent, the quantity of crystalline hormone would have been entirely inadequate for the identification of a new and unknown compound.

Actually we have used about 400 liters of liquor folliculi in our work. Since unselected ovaries from the packing plants yield about 50 cc. of liquor folliculi per pound, 4 tons would be required to obtain 400 liters. However, owing to the cooperation of some of the packing plants which permitted selection of ovaries, we have not aspirated the full 4 tons.

We attempted to use hashed ovaries but owing to the increased difficulties of extraction and purification decided against this procedure. Although the aspiration of the liquor is laborious, it is really an important step in purification and, furthermore, the discarding of the residual ovary is probably not an important source of loss of the follicular hormone (see Doisy, Ralls, Allen, and Johnston, 1924).

In a preliminary paper (MacCorquodale, Thayer, and Doisy, 1935) we reported the isolation of an active principle of sow ovaries. The compound was obtained as the crystalline *m*-bromobenzoate which had the same melting point as an authentic specimen of dihydrotheelin *m*-bromobenzoate. The derivative was hydrolyzed and the hormone itself recovered in a crystalline condition. The melting point agreed with that of dihydrotheelin and a mixed melting point showed no depression. Furthermore, the assays of the hormone and of dihydrotheelin gave identical values.

Although this evidence seemed satisfactory to us, it was realized that more convincing proof was desirable. Since our yields had

been rather disappointing at times, it seemed that we might economize by studying the derivatives of dihydrotheelin. Accordingly, a number of derivatives were made and their solubilities studied. The most suitable compound for our work seemed to be the di- α -naphthoate and, as can be seen from a later section, this derivative was used rather advantageously.

The next attempts at purification were conducted on synthetic dihydrotheelin. The inactive oil recovered from earlier fractionations was added to small quantities of dihydrotheelin and the stability of the latter studied under various conditions. It was found that a quantitative recovery could be obtained by sublimation *in vacuo* of the acetylated mixture. Furthermore, dihydrotheelin was readily isolated in good yield from the hydrolyzed sublimate through the di- α -naphthoate.

The last phase of our isolation experiments was effected on the extract of 107 liters of liquor folliculi plus 56,000 rat units of the partially purified hormone left from our previous work. The total amount of hormone in the 107 liters was about 128,000 rat units, of which about 15 per cent was lost in the preliminary purification, leaving about 109,000 units in a form similar in purity to the 56,000 units saved from earlier work. This total of 165,000 rat units is equivalent to approximately 10.0 mg. of dihydrotheelin.

EXPERIMENTAL

Preparation of Derivatives Used in Our Experiments on Isolation; Dihydrotheelin—The reduction of the carbonyl group of theelin to a secondary alcohol was reported by Schwenk and Hildebrandt (1933) and by Girard, Sandulesco, and Fridensen (1933). The former investigators do not state the method used but they succeeded in obtaining both of the theoretically possible stereoisomers. The latter investigators carried out the reduction by means of sodium in alcohol, and also catalytically by means of nickel in the cold. By both methods they obtained only the isomer melting at 174° (corrected). Laqueur, David, and de Jongh (1935) have also prepared this isomer by means of sodium and alcohol and Danielli, Marrian, and Haslewood (1933) obtained it by a catalytic reduction with platinum. For our work we used the platinum oxide-platinum black catalyst of Adams, Voorhees, and Shriver (1932) in alcohol and obtained practically a quantitative yield of the

lower melting isomer which after crystallization from dilute alcohol melted at 173°.¹ Dirscherl (1936), using the same catalyst, has recently reported results in agreement with ours.

Preparation of m-Bromobenzoates of Dihydrotheelin and Theelin—Dihydrotheelin (21.9 mg.) was dissolved in 10.0 cc. of 10 per cent sodium hydroxide and 4.0 cc. of water. To the solution 0.25 cc. of *m*-bromobenzoyl bromide was added and the flask was shaken vigorously until the reaction was complete. The ester was filtered off, washed with water, and then dissolved through the filter with acetone. It was crystallized once from 70 per cent alcohol, once from dilute acetone, and twice from 95 per cent alcohol. The yield was 27.3 mg. of pure white crystals melting at 155–156°. The analysis showed it to be the mono-*m*-bromobenzoate. The yield was 74.6 per cent of the theoretical.

$C_{25}H_{27}O_3Br$.	Calculated.	C 65.91,	H 5.98,	Br 17.56
	Found.	" 65.93,	" 5.77,	" 17.75, 17 60

From 30.0 mg. of theelin by the same procedure there were obtained 45.6 mg. of *m*-bromobenzoate (90.6 per cent of theoretical) after two recrystallizations from alcohol. Theelin *m*-bromobenzoate crystallizes in stout, well formed needles melting at 221.5–223°.

$C_{26}H_{29}O_3Br$.	Calculated.	C 66.21,	H 5.56,	Br 17.64
	Found.	" 66.13, 66 38,	" 5.47, 5.61,	" 17.55

Preparation of α -Naphthoates of Dihydrotheelin and Theelin—Dihydrotheelin (10.1 mg.) was heated for 1 hour with an excess of α -naphthoyl chloride in 2.0 cc. of dry pyridine. In order to facilitate the removal of the excess acid chloride which reacts extremely slowly with water or dilute alkali at room temperature, an excess (0.5 gm.) of glycine was added and the mixture heated for 1 hour. In this way the water-insoluble α -naphthoyl chloride was converted to α -naphthoylglycine which readily dissolved when the reaction mixture was diluted with a saturated solution of sodium bicarbonate. The insoluble dihydrotheelin dinaphthoate was removed by extraction with ether, the ether was distilled, and the residue dissolved in acetone and treated with norit. The filtrate

¹ All melting points are uncorrected and were taken with a Bureau of Standards calibrated, long stem thermometer.

was diluted with 95 per cent alcohol and heated on the water bath to remove the acetone. It was concentrated until crystals began to separate and was then allowed to stand at 5°. Two recrystallizations gave 15.2 mg. (71 per cent of the theoretical) of white crystals melting at 195–196°.

$C_{10}H_{10}O_4$	Calculated.	C 82 72,	H 6 25
	Found.	" 82 71, 82 81,	" 6 02, 6 16

By the same procedure theelin α -naphthoate was obtained as pure white crystals melting at 200.5–202°.

$C_{11}H_{11}O_4$	Calculated.	C 82 03,	H 6 65
	Found	" 82 02, 82 04,	" 6 58, 6 41

Attempts to prepare the di-*m*-bromobenzoate of dihydrotheelin by this procedure resulted only in the formation of a mono-*m*-bromobenzoate identical with the one obtained by the Schotten-Baumann reaction.

Preparation of Extracts of Liquor Folliculi—Liquor folliculi was aspirated from sow ovaries and preserved with 2 volumes of 95 per cent ethyl alcohol. This mixture was heated to boiling, filtered, and the material remaining on the filter transferred to a beaker and extracted three times with boiling alcohol. The filtrates and extracts were concentrated to dryness by distillation *in vacuo* and the residue emulsified in a little hot dilute sodium hydroxide. This emulsion was extracted with ethyl ether (peroxide-free); the extract was washed with a solution of sodium carbonate and water and distilled. The residue, composed largely of cholesterol and its esters, was partitioned between 70 per cent ethyl alcohol and petroleum ether, as described in an earlier publication (Ralls, Jordan, and Doisy, 1926). The alcoholic solution containing most of the hormone was distilled to dryness, the residue dissolved in a small volume of butyl alcohol (20 cc. for the extract of 8 to 16 liters of liquor folliculi), 5 volumes of petroleum ether were added, and the solution was repeatedly extracted with small volumes of 0.25 N NaOH (see Veler, Thayer, and Doisy, 1930). The alkaline solution was passed through a sintered glass filter and then enough hydrochloric acid added to produce a faint haziness. Exhaustive ether extraction removed the hormone. The ether was distilled, the residue dissolved in toluene, and the

hormone removed from toluene by shaking repeatedly with 0.2 N NaOH containing enough salt to prevent emulsions from becoming troublesome. The alkaline solution was filtered, partially neutralized, and extracted with ether. The ethereal solution was distilled, the residue leached with benzene, and the leachings filtered. The weight of the hormone in this fraction is approximately one-fourth of the total solids.

Isolation of Hormone As m-Bromobenzoate—It was found that upon subjection of an active extract to distillation at 0.02 mm. pressure very little activity was obtained in the distillate collected below 95° but that sublimation of the active material took place rapidly at 130–140°. Three preparations, containing a total of 32,000 rat units, were separately fractionated at 0.02 mm., each being divided into a volatile portion collected below 95° and containing little activity, a middle fraction distilling mainly at 130–140° and containing most of the active substance, and an inactive fraction which did not distil below 150°. The three middle fractions which had a combined weight of 4.23 mg. were dissolved in 100 cc. of 65 per cent alcohol (by volume) and shaken with 20 cc. of benzene. The alcoholic solution was evaporated to dryness and the residue (3.38 mg.) was dissolved in 1.0 cc. of benzene and filtered from a small amount of insoluble material. The solution was evaporated to a volume of 0.3 cc. and 1 cc. of petroleum ether (b.p. 30–60°) was added. The solution was cooled to 5° and the white precipitate filtered off and washed with petroleum ether. It was dissolved in 95 per cent alcohol, concentrated to 1.5 cc., and a few drops of water were added. On standing overnight at 5° the material crystallized from the solution in fine white needles which were filtered off and washed with water. The weight of the crystals was 1.05 mg. and the bioassay showed the rat unit to be 0.165 microgram (Preparation 2-M-16).

Another extract (weight 2.26 mg.) containing 8000 rat units was carefully and repeatedly fractionated at 0.02 mm. pressure and a white crystalline sublimate obtained which weighed 1.73 mg. The bioassay showed it to be of approximately the same degree of purity as Preparation 2-M-16 (0.165 microgram) and so the two were combined for further treatment. The precipitation from benzene by means of petroleum ether was repeated and the material then was combined with Preparation 2-M-26 (weight 2.05

mg.) which contained 26,000 rat units and had been purified in the same manner as Preparation 2-M-16. The total weight of these combined preparations obtained from 70 liters of liquor folliculi was 4.83 mg., with an activity of 43,000 rat units. It was sublimed carefully at a pressure of 0.02 mm. by heating at 110–115° for 48 hours. The sublimate was nicely crystalline and white with a fringe of clear, glassy material. The melting point of the crystalline portion was 162–163.5°. The weight was 3.48 mg. and the rat unit 0.08 microgram. The material was dissolved in 4 cc. of 10 per cent sodium hydroxide solution and 2 cc. of water, and shaken vigorously with 0.1 cc. of *m*-bromobenzoyl bromide. When the reaction was complete, the precipitate was filtered off and washed with water. It was dissolved in acetone and alcohol and filtered. The filtrate was concentrated to 0.25 cc. and allowed to stand overnight at 5°. The crystals which separated were filtered off, washed with methyl alcohol, and dried at 110°; weight 2.6 mg. The product was recrystallized by dissolving in acetone, diluting with 95 per cent ethyl alcohol, concentrating to 0.25 cc., and allowing to stand at 5°. As the material thus obtained was still slightly yellow, it was dissolved in acetone and treated with norit. Crystallization from 95 per cent alcohol then gave 1.3 mg. of pure white fluffy crystals identical in appearance with the *m*-bromobenzoate of dihydrotheelin. The melting point was 154–155°.

The ester was hydrolyzed by heating for 1 hour in a platinum vessel with 2 cc. of 5 per cent alcoholic potassium hydroxide. The solution was diluted with water, heated to remove the alcohol, acidified to Congo red with hydrochloric acid, and allowed to stand at 5° overnight. The precipitate was filtered off, washed with sodium bicarbonate solution and then with water, and dried at 110°; weight 0.45 mg. It was dissolved in 0.4 cc. of 95 per cent alcohol, treated with a few grains of norit, and filtered. A few drops of water were added to the filtrate, which was then concentrated to 0.2 cc. on the water bath and allowed to stand at 5°. The hormone crystallized in small white needles (see Fig. 1) identical in appearance with those of dihydrotheelin obtained from theelin. The crystals were filtered off, washed with water, and dried at 110°; weight 0.35 mg. The melting point was 170–171°. A mixed melting point with dihydrotheelin showed no depression. The

bioassay of the hormone crystals showed the rat unit to be 0.06 microgram.

Isolation of the Hormone As the Di- α -Naphthoate—Preparation 177-d (76,000 rat units) was refluxed for 1 hour with 1 cc. of acetic anhydride. The excess acetic anhydride was removed by distillation and the brown glassy residue subjected to distillation at 0.02 mm. pressure. Two fractions were obtained, one distilling mainly at 95–105° and the other at 135–150°. The latter was

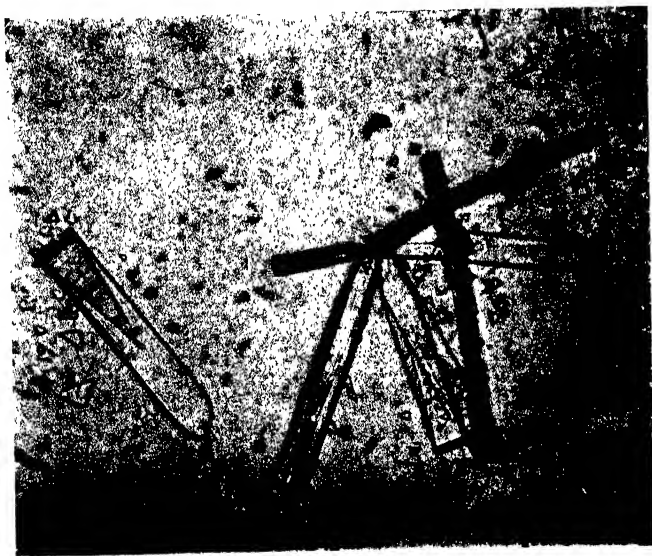


Fig. 1. $\times 290$. Dihydrotheelin from the ovary. The hormone crystallizes in needles and in platelets, both of which appear in the photomicrograph.

entirely oily but the former contained a considerable quantity of well formed white crystals. This fraction was carefully refractionated and a pure crystalline sublimate obtained at 65–70°, which, however, proved to have no estrogenic activity. The amount was small and it has not yet been investigated. As the fractionation was continued, the temperature was raised to 130–135° and a clear, colorless, oily distillate of the hormone acetate was obtained. This fraction, together with the first one ob-

tained at 135–150°, was hydrolyzed with alcoholic potassium hydroxide, and the solution acidified with hydrochloric acid and extracted thoroughly with ether. Distillation of the ether left a pale yellow oil (8.60 mg.; the rat unit was 0.130 microgram). The oil was dissolved in alcohol and heated with semicarbazide acetate for 1½ hours. The solution was evaporated to dryness and leached alternately with benzene and water. The insoluble fraction thus obtained weighed 1.33 mg. but it contained practically no theelin semicarbazone for after hydrolysis with hydrochloric acid the bioassay showed no significant increase. By control experiments it had been found that 0.5 mg. of theelin could be easily separated in this way. From our investigations it appears that if there is any theelin in the follicular fluid of sow ovaries, it accounts for only an insignificant fraction of the estrogenic activity.

The benzene-soluble fraction was dissolved in alcohol and the solvent allowed to evaporate spontaneously. A residue remained which was partly crystalline and partly oily. The oily droplets were washed from the crystalline material by means of 1.0 cc. of benzene. The crystals remaining in the tube were sublimed at 0.02 mm. and 160°; weight 1.875 mg.

Preparation 185-c-11 (12.5 mg.; 54,000 rat units) was purified by fractional distillation of the acetate, followed by hydrolysis and sublimation *in vacuo*. In this way 5.15 mg. of crystals were obtained. This material was combined with the 1.875 mg. of crystals of Preparation 177-d and dissolved in 2.0 cc. of dry pyridine. To this solution 0.10 cc. of α -naphthoyl chloride was added and the solution heated for 1 hour on the water bath. Then 0.5 gm. of glycine was added and the mixture heated on the water bath for 1 hour, after which the reaction mixture was diluted with an excess of saturated sodium bicarbonate solution. The precipitate which remained was taken up by shaking with ether. Distillation of the ether extract produced a residue of yellow oil containing fine white crystals. This was dissolved in acetone, treated with norit, and filtered. The filtrate was diluted with alcohol and the acetone removed by boiling. The alcoholic solution was concentrated to 1.5 cc. and allowed to stand at 5°. Crystals of the very sparingly soluble dihydrotheelin dinaphthoate separated, leaving practically all of the contaminating substances in the

mother liquor. The crystals were filtered off, washed with methyl alcohol, and recrystallized by the same procedure. After drying at 110° the crystals, which had a faint yellow color, weighed 7.8 mg. They were dissolved in acetone, treated with norit, and recrystallized from 95 per cent alcohol, producing 7.0 mg. The fourth recrystallization gave 6.4 mg. of pure white crystals which melted at 191–193°. A specimen of pure dinaphthoate of dihydrotheelin which had been made from theelin melted at 194–195° (taken simultaneously in the same bath) and the mixed melting point was 192–194°. The melting point of a mixture of the naphthoates of dihydrotheelin and theelin was 175–180°.

Preparation 189-c-2 (12.5 mg.; 35,000 rat units) was combined with the benzene washings of the impure crystals obtained from the preceding extract (Preparation 177-d) and purified by the process used for Preparation 185-c-11. After four recrystallizations from 95 per cent alcohol there were obtained 4.6 mg. of pure dinaphthoate of the hormone with a melting point of 191–192.5°. On mixing with pure dihydrotheelin dinaphthoate the melting point was not depressed. The total amount of pure hormone dinaphthoate obtained from 165,000 rat units (\approx 10.0 mg. of dihydrotheelin) was thus 11.0 mg., which corresponds to a recovery of 52 per cent. Of this, 1.243 mg. were used for a molecular weight determination (Rast) and combustion analysis (Pregl).

$C_{40}H_{36}O_4$.	Calculated.	C 82.72,	H 6.25,	mol. wt. 580
	Found.	" 82.35,	" 6.24,	" " 572

The remaining hormone dinaphthoate (9.8 mg.) was saponified by refluxing with alcoholic potassium hydroxide and the solution diluted with water and acidified with hydrochloric acid. An excess of sodium bicarbonate was added and the cold solution extracted five times with ether. The extract was washed with water and the ether distilled, leaving a white crystalline residue which was dissolved in methyl alcohol, treated with norit, and crystallized from 1.5 cc. of dilute methyl alcohol. The weight of the pure white needles was 3.5 mg. and the melting point 171–172°. A specimen of dihydrotheelin beside it on the thermometer melted at 172–173°. The melting point of a mixture of the two was 171.5–173°. The melting point of a 3:1 mixture of dihydrotheelin and theelin was 164–168°. Of the 3.5 mg. of hormone 2.030 mg. were

used for a molecular weight determination and two combustion analyses.

$C_{15}H_{24}O_2$.	Calculated.	C 79.36,	H 8.88,	mol. wt. 272
	Found.	" 79.12, 79.27,	" 8.91, 8.79,	" " 267

Bioassay—Since the establishment in 1932 of the "international standard" for the assay of the follicular hormone, we have conducted many experiments in a study of factors affecting the accuracy of assays. This work, which is now being prepared for publication, has indicated some of the precautions to be observed to obtain accurate results. We are convinced that in order to secure consistent results, uniformity of past history of animals is essential. Furthermore, for the greatest precision we find it necessary to conduct a parallel assay of the standard preparation on the same day with a group of comparable animals. In assaying this crystalline material from the liquor folliculi we have carried out on the same day parallel assays of dihydrotheelin prepared from theelin.

Modified Marrian-Parkes Procedure—Ovariectomized mice were given subcutaneous injections of four equal quantities of an aqueous solution at intervals of 12 hours.

<i>Assay</i> —Dihydrotheelin	200,000 units per mg.
Crystals from ovary	200,000 " " "

Modified Butenandt Procedure—A single subcutaneous injection of ovariectomized mice with the substance dissolved in oleum sesami was made.

<i>Assay</i> —Dihydrotheelin	67,000 units per mg.
Crystals from ovary	67,000 " " "

Modified Allen-Doisy Procedure—Ovariectomized rats were given subcutaneous injections of three equal quantities of an aqueous solution at intervals of $4\frac{1}{2}$ hours.

<i>Assay</i> —Dihydrotheelin	16,600 units per mg.
Crystals from ovary	16,600 " " "

Curtis-Doisy Procedure—Immature female rats were given subcutaneous injections of an aqueous solution in the morning and evening of 3 successive days beginning at 18 days of age.

<i>Assay</i> —Dihydrotheelin	5000 units per mg.
Crystals from ovary	5000 " " "

The identity of the active principle isolated from the Graafian follicle with dihydrotheelin prepared in the laboratory from theelin of pregnancy urine is thus established.

DISCUSSION

Our first isolation of the hormone as the *m*-bromobenzoate was preceded by a series of careful and time-consuming fractional sublimations *in vacuo*. Although the pure crystalline hormone sublimes unchanged under the conditions of our experiments, its separation from the other substances present in the extracts cannot be accomplished by this method without considerable loss of activity. It was subsequently found that acetylation of the extract before fractionation greatly increased the stability of the hormone and permitted an easy separation of the diacetate by one or two fractionations.

The later work in which the naphthoate was employed gives an over-all yield of approximately 50 per cent. which is much more satisfactory than was obtained with the bromobenzoate. While the *m*-bromobenzoyl bromide reacts with crystalline dihydrotheelin to give a good yield it cannot be advantageously employed to separate the hormone from the impurities present in the extract. The solubilities of the ester and the impurities are such that the separation is unsatisfactory. On the other hand, the slight solubility of the dinaphthoate in ethyl alcohol permits a ready separation of the hormone from contaminating substances.

Shortly after our announcement of the isolation of dihydrotheelin from sow ovaries, Wintersteiner, Schwenk, and Whitman (1935) reported the isolation of the same substance and its isomeric form from the urine of pregnant mares. These two investigations are the only reports on the occurrence of natural dihydroxy estrogenic substances.

Though we have obtained a large proportion of the estrogenic substances of sow ovaries as crystalline dihydrotheelin, this finding does not exclude the presence of other estrogenic compounds. However, measured in physiological potency the amount of such compounds is small. One would expect the presence of other active compounds, since it is highly probable that some of the compounds of the intermediate steps in the formation by the ovary would possess some activity.

The isolation of dihydrotheelin from sow ovaries does not permit us to say that the ovaries of other species contain this hormone. One needs only to recall the difference between the estrogenic substances of human and mare urine to realize the danger of exceeding the data in drawing conclusions in this field. However, in spite of the limitation mentioned the isolation of the principal estrogenic compound of sow ovaries is a source of satisfaction, since it represents the conclusion of work begun by one of us in 1923.

Assay by the vaginal smear method shows that dihydrotheelin is more potent than any other known substance in producing cornification in the vagina of the ovariectomized rat or mouse. Again, it would be unwise to exceed our data. This compound may be less active if assayed by other types of response produced by estrogenic substances.

Since the statements of the large number of rat units used may convey an erroneous impression to those not active in this field it should be stated that the total amount of estrogenic substance, calculated as dihydrotheelin, in the liquor folliculi of a ton of sow ovaries is about 6 mg. The concentration in the liquor folliculi is about 1 part in 15,000,000 and in the ovary of the sow 1 part in 150,000,000.

SUMMARY

1. Dihydrotheelin has been isolated from the liquor folliculi of sow ovaries as the *m*-bromobenzoate and also as the di- α -naphthoate. Both derivatives were obtained as pure crystalline compounds agreeing in melting points with the corresponding derivatives prepared from dihydrotheelin obtained from theelin. A mixed melting point determination on the naphthoates showed no depression. The derivatives were hydrolyzed and the hormone obtained in the pure crystalline condition. In each case the melting point was in agreement with that of dihydrotheelin prepared from theelin, and the mixed melting point showed no depression.

2. Complete analytical data, as well as molecular weight determinations, have been obtained for the naphthoate and for the hormone obtained from it by hydrolysis.

3. Bioassay of the hormone by four standardized procedures shows it to have the same estrogenic activity as dihydrotheelin prepared in the laboratory.

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FURTHER EVIDENCE FOR THE ABSENCE OF ALLOCHOLESTEROL FROM THE ORGANISM*

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The fact that allocholesterol yields coprosterol on hydrogenation led several workers to suggest that it is concerned in the biological transformation of cholesterol to coprosterol (1), although recent evidence indicates that the conversion involves the ketones, cholestenone and coprostanone, as intermediates (2). Schoenheimer, Dam, and von Gottberg (3) investigated the presence of allocholesterol in the organism, using the carmine-red color of the Rosenheim reaction with trichloroacetic acid as a means of quantitative assay. With this method they could find no evidence for the presence of allocholesterol in the various tissues investigated with the exception of the sterols from hen's eggs. The Rosenheim reaction here was faintly positive. In view of the unspecific nature of the color test the question was left open as to whether the positive reaction obtained with the egg sterols represented a small amount of allocholesterol or not.

We have recently reported the preparation of pure allocholesterol by the reduction of cholestenone with aluminum isopropylate, the compound being especially characterized by its quantitative dehydration on acid treatment to the doubly unsaturated Δ -2,4-cholestadiene (4). The specific absorption in the ultraviolet region shown by this hydrocarbon permits its ready detection in very small amounts. It is possible, therefore, to use this new reaction as a means of determining allocholesterol in the presence of

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other digitonin-precipitable sterols, since none of the known precipitable sterols or their derivatives is dehydrated under the conditions of acid treatment.¹ We have applied this method to the still unsettled question of the possible presence of small amounts of allocholesterol in the sterols from hen's eggs.

The application of the method to this material gave negative results, the sensitivity of the method being such as to permit the detection of less than 0.01 per cent allocholesterol.

EXPERIMENTAL

The yolks of eleven fresh eggs were stirred into 500 cc. of 25 per cent aqueous KOH and the solution let stand for 72 hours at room temperature in a nitrogen atmosphere. Water was then added and the material extracted with peroxide-free ether. The ether was removed at room temperature and the residue dissolved in 500 cc. of 10 per cent KOH in methyl alcohol. After standing at room temperature in a nitrogen atmosphere for 60 hours the solution was diluted and extracted with ether. The ether extract was washed and dried over Na_2SO_4 and K_2CO_3 and the ether removed at room temperature, a yellow crystalline residue of 2.8 gm. being obtained. 1 gm. of this material was dissolved in 200 cc. of 80 per cent alcohol and a solution of 5 gm. of digitonin in alcohol added. The digitonides (4.96 gm.) were filtered off and the sterols regenerated by dissolving in cold pyridine and precipitating the digitonin with ether (5). The sterols thus obtained were dissolved in 10 cc. of ether, an equal volume of methyl alcohol added, and the ether removed in a stream of carbon dioxide. The white crystalline material thus obtained gave a faintly positive Rosenheim reaction and showed no absorption in the ultraviolet region. 100 mg. of this material were dissolved in 10 cc. of 95 per cent ethyl alcohol, 2 drops of concentrated HCl added, and the solution refluxed for 2 hours. Water was then added, the sterols extracted with ether, and the ether, after washing, removed. The residue still gave a positive Rosenheim reaction; the absorption spectrum in the ultraviolet region showed no change. 99 mg. of this acid-treated material were dissolved in alcohol and an excess of digitonin

¹ Epiallocholesterol would likewise dehydrate to Δ -2,4-cholestadiene under the conditions of acid treatment (4). Its presence is eliminated, however, by the initial digitonin precipitation.

was added. The digitonides (399 mg.) were filtered off, and the filtrate was taken to dryness *in vacuo*. The residue was repeatedly extracted with ether, and the ether extracts united and taken to dryness. The small quantity of residue was dissolved in 10 cc. of 95 per cent alcohol and its ultraviolet absorption spectrum investigated. There was no indication of the presence of the absorption bands of Δ -2,4-cholestadiene (no maxima at 229, 235, and 240 $m\mu$). Further, the digitonides from the acid-treated material gave the faint Rosenheim reaction, while the non-precipitable fraction gave no color with trichloroacetic acid.

SUMMARY

The quantitative dehydration of allocholesterol to Δ -2,4-cholestadiene on acid treatment and the specific ultraviolet absorption spectrum shown by the latter compound permit the use of this reaction as a method for determining allocholesterol in the presence of other digitonin-precipitable sterols. In applying this method to the sterols of hen's eggs no indication of the presence of allocholesterol could be found.

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FURTHER STUDIES ON THE AVAILABILITY OF COPPER FROM VARIOUS SOURCES AS A SUPPLEMENT TO IRON IN HEMOGLOBIN FORMATION*

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In a previous paper on this subject (1) it was shown that the copper of copper caseinate, glycine amide biuret, alanine amide biuret, hemocyanin, and of whole wheat was readily utilized by severely anemic rats to supplement iron for hemoglobin formation. The copper of copper hematoporphyrin, however, was found to be not available even when fed at high levels. This indicated that copper may exist in a form which cannot be utilized by the animal and that possibly animal and plant tissues might contain at least some of the copper in unavailable form. The implications of this were discussed in our earlier paper. These studies have since been extended to include a few more animal and plant tissues as well as some compounds in the form of which copper might occur in biological materials.

EXPERIMENTAL

The experiments were carried out as previously described. Milk plus 0.5 mg. of purified iron daily constituted the basal diet of the anemic rats to which such amounts of the various sources of copper were added as to supply 0.01 mg. of Cu daily. The copper compounds studied were mixed with sufficient purified starch to permit accurate weighing of the daily dose. In all cases the supplements were fed in the morning with a small amount of milk to

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insure rapid and complete consumption. Milk *ad libitum* was given later in the day.

As usual we observed great precautions to avoid contamination with ionic copper. Copper citrate (Abbott) and copper nucleinate (Parke, Davis and Company) were dialyzed for 48 hours against redistilled water before they were used for the experiment. Similarly the copper aspartate, copper pyrophosphate, and cysteine cuprous mercaptide (the latter prepared according to Vickery and White (2), avoiding an excess of copper) which we prepared were dialyzed against 0.1 per cent H_2SO_4 in redistilled water for 48 hours. Control animals were fed 0.5 mg. of Fe daily or 0.5 mg. of Fe plus 0.01 mg. of Cu as $CuSO_4$. The rats receiving pure iron only died from anemia in all cases. Hemoglobin determinations were made weekly by the Newcomer method.

DISCUSSION

The results are represented in Chart I. From these curves it is evident that the copper from all sources supplied was readily available to supplement iron in hemoglobin formation. In general the rate and extent of hemoglobin regeneration were about the same in all cases and corresponded closely with those obtained when 0.01 mg. of Cu was fed daily as copper sulfate. When wheat germ was fed as the source of copper, the response during the 1st week was greater than is usually observed with this level of copper. Possibly the additional vitamin supply carried by the wheat germ (1.43 gm. were fed daily) was responsible for this. The responses observed with dried pork heart and dried pork liver were somewhat slower and less extensive. This may partly be due to the fact that in the early stages of the experiment the rats sometimes failed to consume the entire supplement and that their daily copper intake was actually a little less than 0.01 mg. It is also possible that the slightly poorer utilization of copper that we observed when pork heart or pork liver was used as source of copper is similar to the decreased copper storage in the liver, which Coulson *et al.* (3) observed as a result of adding meat scraps to a diet high in copper. If, however, some of the copper of pork heart or pork liver was actually present in unavailable form, it would constitute such a small portion of the total copper as to be of no practical consideration.

Of particular interest is the fact that the copper of brewers' yeast was wholly available. Fischer and Fink (4) isolated considerable amounts of copper coproporphyrin from the yeast protein, cymo-

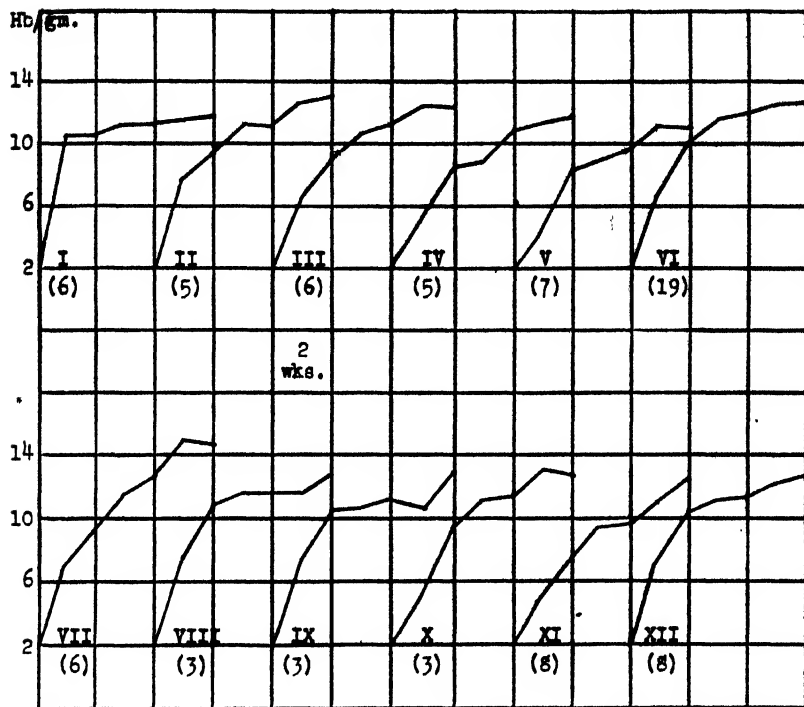


CHART I. Availability of copper for hemoglobin formation. Composite curves of hemoglobin regeneration of rats receiving 0.5 mg. of Fe daily plus 0.01 mg. of Cu from: Curve I, wheat germ; Curve II, alfalfa; Curve III, Anheuser-Busch yeast; Curve IV, dried pork heart; Curve V, dried pork liver; Curve VI, copper sulfate; Curve VII, cysteine cuprous mercaptide; Curve VIII, copper aspartate; Curve IX, copper citrate; Curve X, copper nucleinate; Curve XI, copper pyrophosphate; Curve XII, copper sulfate (curve reported 1934). The number of animals in each group is indicated in parentheses.

casein. They express the opinion, however, that the copper porphyrin was not preformed in the yeast cell but that it was formed during the isolation of the porphyrin due to copper contamination.

Similar conclusions were reached by Stone and Coulter (5) who had evidence of the occurrence of copper coproporphyrin in extracts from bacteria.

Our results indicate that the yeast we used did not contain copper porphyrin and that in the digestive tract there was no formation of a complex between copper and porphyrin of the yeast. Living organisms evidently do not require the presence of appreciable quantities of copper porphyrins.

Although the other copper compounds that were used in this study are quite insoluble, they were readily utilized by the anemic rats. The fact that copper fed with milk, copper caseinate, biuret compounds, copper aspartate, and cuprous cysteine mercaptide are readily utilized indicates that the formation in the intestinal tract of copper complexes by proteins or their digestion products does not interfere with absorption of copper. Brand and Stucky (6) found that copper glutamate is readily utilized by anemic rats. Handovsky (7) reported a marked hematogenic effect of "copper protein" and copper tyrosine. Keil and Nelson (8) found that anemic rats could utilize Cu_2O , $\text{Cu}(\text{OH})_2$, and CuI . Oda (9) observed the same metabolic effects when copper was fed as copper nitrate, copper proteinate, or copper glycerophosphate. Feeding copper as a "siderac-copper compound" prepared by alkaline precipitation of ferric or ferrous iron and copper sulfate and heating the precipitate at 300° in oxygen, failed, however, to produce the effects observed with the other copper compounds. But it is unlikely that copper occurs in foodstuffs in such a form.

Besides copper porphyrin the only copper compound which has been found to be a poor source of copper is CuS . Sherman, Elvehjem, and Hart (10) reported that the incomplete hemoglobin regeneration observed when egg yolk was used as a source of iron was "not due to the unavailability of the iron present but to a retarded utilization of the copper" and that this may be related to the formation of insoluble copper sulfide in the digestive tract. When they fed 0.01 mg. of Cu daily as CuS (taking precautions to avoid oxidation of the sulfide) to anemic rats they observed only slight hemoglobin regeneration. Keil and Nelson (8) also observed that CuS is less efficiently used than other copper salts. In contrast to this, organic sulfide derivatives are available, as our results with cysteine cuprous mercaptide indicate.

From our work on the availability of copper and the isolated observations of others that we have quoted, it appears that the unavailability of copper in naturally occurring food materials and compounds that might be used for copper therapy is not a problem of practical importance as far as the dietary supply of copper is concerned. Under certain conditions the formation in the digestive tract and the unavailability of CuS may have to be considered as a factor in decreasing the utilization of dietary copper.

CONCLUSIONS

1. The copper of wheat germ, alfalfa, brewers' yeast, pork heart, pork liver, cysteine cuprous mercaptide, copper aspartate, copper citrate, copper nucleinate, and copper pyrophosphate is readily utilized by severely anemic rats to supplement iron for hemoglobin formation.
2. Unavailability of dietary copper is apparently of little practical importance.

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ON THE OXIDATION PRODUCT OF CATECHOL WHEN OXIDIZED BY MEANS OF TYROSINASE

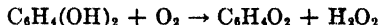
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(Received for publication, June 12, 1936)

It was only natural that the earlier investigators of the action of the oxidase, tyrosinase, on catechol should have assumed that the first stage in the reaction is the formation of *o*-quinone. More recently this view of the reaction has gained additional approval from the studies carried on by Pugh and Raper (1). One of the difficulties encountered in trying to determine the nature of the initial oxidation product of the catechol is due to the great instability of *o*-quinone, especially when the latter occurs in an aqueous solution. Since *o*-quinone forms with aniline an insoluble dianilinoquinone, Pugh and Raper allowed the enzymatic oxidation of catechol to take place in the presence of aniline. The formation of the dianilinoquinone thus obtained is regarded by them as evidence that *o*-quinone is the initial oxidation product in the reaction.

About 2 years previous to Pugh and Raper's publication, Robinson and McCance (2) showed that in the oxidation of catechol by means of tyrosinase, 2 atoms of oxygen are used up per mole of catechol. Since only 1 atom of oxygen is required in converting catechol into *o*-quinone, the question naturally arises as to what becomes of this second atom of oxygen. Onslow and Robinson (3) have suggested that the oxidation of the catechol to *o*-quinone is accompanied by the formation of hydrogen peroxide.



However, their attempts to prove the formation of hydrogen peroxide in the reaction do not seem to be very convincing. Pugh and Raper expressed doubt concerning the formation of hydrogen peroxide, pointing out that some of their preparations of tyrosin-

ase also contained catalase. The presence of the latter would obviously tend to decompose any peroxide formed, thereby returning the second atom of oxygen to the reaction mixture. In order to reconcile their claim that *o*-quinone is the oxidation product of

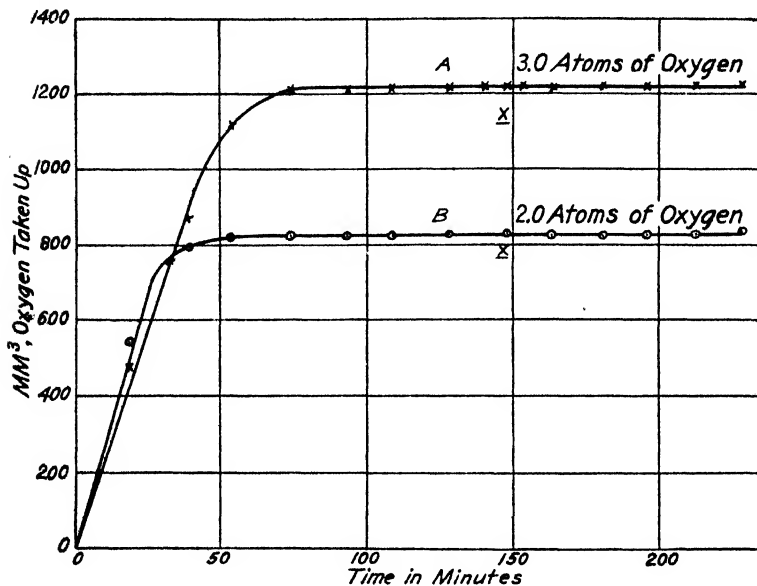
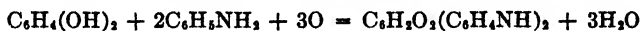


FIG. 1. Showing that 3 atoms of oxygen per mole of catechol were required in the formation of the dianilinoquinone. Curve A, the reaction mixture = 3 cc. of 0.75 M citrate-phosphate solution + 2 cc. of a 3 per cent aqueous aniline solution + 2 cc. of 0.2 per cent aqueous catechol solution + 1 cc. of tyrosinase solution (13.5 units); pH 6.0. Curve B, the reaction mixture is the same as that corresponding to Curve A except 2 cc. of water were used in place of the 2 cc. of the 3 per cent aqueous aniline solution; pH 6.0; temperature 25°. At points marked X, 6.5 more units of enzyme were added. 407 units on the ordinate axis correspond to 1 atom of oxygen. The enzyme preparation was obtained by precipitating the pressed juice from mushrooms with 3 volumes of cold acetone, dissolving the precipitate in water, and dialyzing against cold distilled water for 3 days.

catechol with the consumption of 2 atoms of oxygen per mole of catechol, they suggest that in the absence of aniline the quinone suffers further oxidation to a higher oxidation product, the chemical nature of which is still undetermined.

In going over Pugh and Raper's report on their study, the pres-

ent authors were unable to find any direct evidence supporting their suggestion that it is the *o*-quinone, rather than the more highly oxidized product from the catechol, that reacts with the aniline to form the dianilinoquinone. Obviously the formation of the dianilino compound, whether from *o*-quinone and aniline or from a more highly oxidized catechol and aniline, involves still further oxidation. In the present study it has been found that 3 atoms of oxygen are consumed in the oxidation of catechol in the presence of aniline (Fig. 1) and the same dianilino compound is obtained as described by Pugh and Raper.



In the absence of aniline only 2 atoms of oxygen were required to oxidize a mole of catechol, just as had been reported by Robinson and McCance and later by Pugh and Raper. It was also found that the total amount of oxygen consumed, 2 atoms per mole of catechol in the absence and 3 atoms in the presence of aniline, is independent of pH between pH 4.3 and 8. By adding aniline to the reaction mixture, after the catechol had been previously permitted to take up 2 atoms of oxygen per mole, and therefore oxidized to a higher state than *o*-quinone, the same dianilinoquinone was obtained as when the catechol was oxidized in the presence of aniline. On measuring the amount of oxygen required to form the dianilino compound, when the aniline was added to the reaction mixture after the catechol had taken up 2 atoms of oxygen, it was found to correspond only to 1 atom per mole of catechol (Fig. 2).

From the observations cited above it seems just as logical to regard the oxidation of catechol by means of tyrosinase as going directly to the oxidation product resulting from the consumption of 2 atoms of oxygen per mole of catechol, as to adhere to the view held by Pugh and Raper, *viz.* *o*-quinone being formed first and as such reacting with the aniline to form the dianilinoquinone. This new view of the mechanism involved in the enzymatic oxidation of catechol also has the advantage that it takes care of the second atom of oxygen used up in the oxidation of catechol, which has given rise to so much speculation by different workers. Likewise, in line with this view are the observations of Reinders and Dingemans (4). They find that in the oxidation of hydroquinone by oxygen, using a trace of copper salts as catalyst, a hydroxy-*p*-quinone results especially in solutions of pH in the neighborhood of

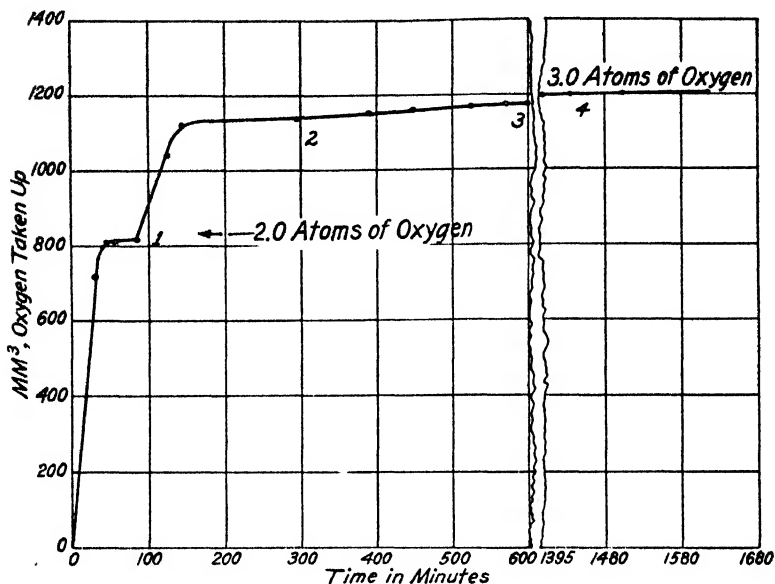
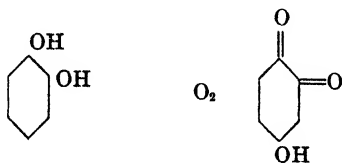


FIG. 2. Showing that only 1 atom of oxygen per mole of catechol was taken up in the formation of the dianilinoquinone, when the aniline was added after the catechol had taken up previously 2 atoms of oxygen. The reaction mixture = 4 cc. of 0.75 M citrate-phosphate solution + 2 cc. of 0.2 per cent aqueous catechol solution + 2 cc. of tyrosinase solution (11.2 units); pH 6.1; temperature 25°. At the point marked 1, 2 cc. of 3 per cent aqueous aniline solution together with 2 cc. of enzyme solution (11.2 units) were added; at points marked 2, 3, and 4, 2 cc. of enzyme solution (11.2 units) were added. The enzyme solution was prepared by removing some extraneous protein from fresh mushroom juice by means of trichloroacetic acid, neutralizing the filtrate to pH 6, and dialyzing for 6 days against cold distilled water.

8. Very likely catechol is oxidized in the presence of tyrosinase and oxygen to a hydroxy derivative of *o*-quinone, and in this way requires the 2 atoms of oxygen per mole.



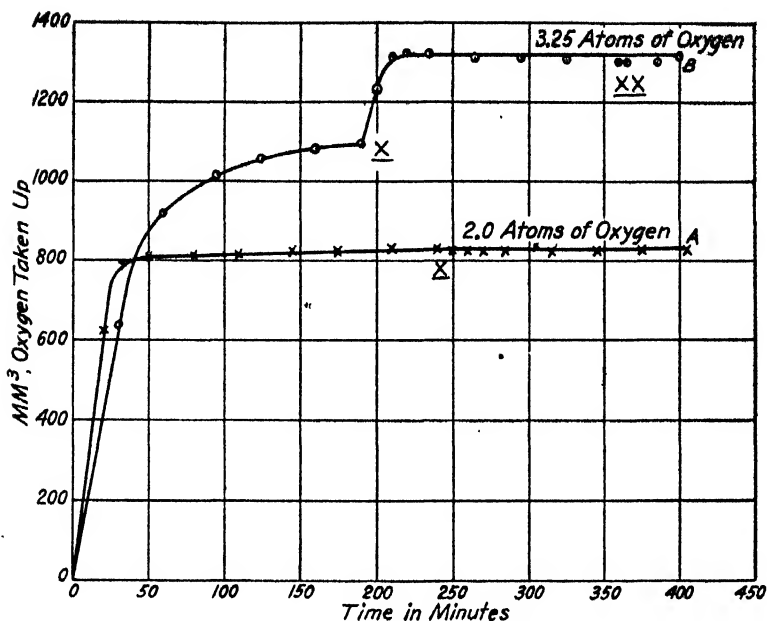
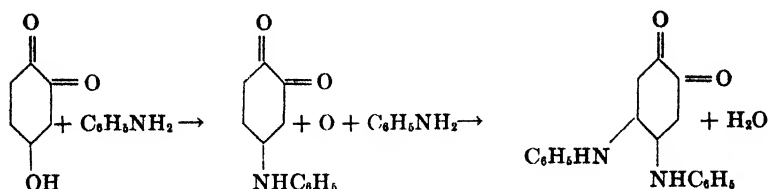


FIG. 3. Showing that tyrosinase exerts a catalytic influence on the reaction involving the third atom of oxygen taken up by catechol in the formation of the dianilinoquinone. Curve A, the reaction mixture = 3 cc. of 0.75 M citrate-phosphate buffer solution + 2 cc. of 0.2 per cent aqueous solution of catechol + 2 cc. of water + 1 cc. of enzyme solution (4.7 units); pH 5.8. Curve B, the reaction mixture is similar to that for Curve A except 2 cc. of 1.5 per cent aqueous solution of aniline were used in place of 2 cc. of water and the 2 cc. of enzyme solution in this case contained only 1.8 units. At points marked X and XX 1.2 additional units of enzyme were added. The pH of the reaction mixtures was 5.8; temperature 25°. 407 units on the ordinate axis correspond to 1 atom of oxygen. The enzyme preparation used was obtained by first treating fresh mushroom juice with a small amount of dilute HCl to remove some of the accompanying proteins; the filtrate obtained was then treated with a small amount of trichloroacetic acid, and the filtrate thus obtained was neutralized to pH 6. The neutral filtrate was then made 0.6 saturated with $(\text{NH}_4)_2\text{SO}_4$, filtered, and the precipitate dissolved in water and dialyzed against distilled water for 2 days.

The formation of the hydroxyquinone also agrees with the fact that only 1 additional atom of oxygen is taken up in the formation

of the dianilinoquinone,¹ when aniline is added to the oxidized catechol.



Although the dianilinoquinone is easily formed, as shown by Pugh and Raper, when an ethereal solution of *o*-quinone is shaken with water containing some aniline, it seems from the rate of oxidation of the catechol in the presence of aniline that tyrosinase also exerts a catalytic influence on the rate at which the third atom of oxygen is taken up in the reaction. Curve B in Fig. 3 represents the uptake of oxygen when catechol was oxidized in the presence of aniline. As has been pointed out by Graubard and Nelson, tyrosinase gradually becomes inactivated in the oxidation of catechol, and hence when only 1.8 units² of the enzyme preparation were used in the beginning of the experiment, corresponding to Curve B, the oxidation had almost ceased at the expiration of 200 minutes (the point marked X on the curve) and only about 2.5 atoms of oxygen were consumed per mole of catechol instead of 3. However, by adding to the reaction mixture 1.2 additional units of the enzyme preparation, oxidation again set in until 3 atoms (3.25) were taken up. Upon further addition of 1.2 units of the enzyme preparation (the point marked XX on Curve B) no further uptake of oxygen occurred, indicating that all the catechol had been oxidized and converted into the dianilinoquinone.

EXPERIMENTAL

The rates of oxidation of the catechol as well as the total amount of oxygen consumed were measured by means of the Barcroft-Warburg respirometer as described by Graubard and Nelson (6). The enzyme preparations used were obtained from the common or field mushroom (*Psalliota campestris*) and the method of preparation was similar to that described by Graubard and Nelson.

¹ The structure assigned to the dianilino-*o*-quinone is that proposed by Kehrmann and Cordone (5).

² For units of tyrosinase see Graubard and Nelson.

SUMMARY

1. The total amount of oxygen required in the formation of the dianilinoquinone from catechol and aniline, in the presence of tyrosinase, is 3 atoms per mole of catechol. This relationship holds over the pH range 4.3 to 7.

2. The total amount of oxygen required in the oxidation of a mole of catechol is 2 atoms over the pH range 4.3 to 8.

3. Only 1 atom of oxygen per mole of catechol is required in the formation of dianilinoquinone, when the catechol is permitted to take up 2 atoms of oxygen previous to the addition of aniline to the reaction mixture.

4. The taking up of the third atom of oxygen in the formation of the dianilinoquinone from catechol and aniline is also catalyzed by the tyrosinase.

5. The experimental evidence up to the present indicates that the initial oxidation of catechol by means of tyrosinase involves 2 atoms of oxygen per mole of the catechol.

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**A COMPARISON OF THE CALORIGENIC POTENCIES
OF *l*-THYROXINE, *dl*-THYROXINE, AND
THYROID GLAND**

**WITH A NOTE ON THE THYROXINE CONTENT OF THE ACID-
SOLUBLE FRACTION OF THE PEPTIC DIGEST OF
THYROID PROTEIN .**

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In a previous paper (1) it was shown that (a) the calorogenic activity of desiccated thyroid preparations is proportional to the thyroxine content of the gland as determined by the method of Leland and Foster (2), and (b) that the calorogenic activity of a certain dosage of thyroxine in the form of thyroid gland is nearly twice as great as the activity of the same dosage of pure racemic thyroxine, when both are administered orally. It was suggested that this difference may be due to the greater biological activity of the naturally occurring *l*-thyroxine. In this paper experiments are presented which show that this is the case, and that the calorogenic activity of a certain dosage of pure *l*-thyroxine is quantitatively equal to the activity of the same amount of thyroxine in the form of thyroid gland substance.

EXPERIMENTAL

Isolation of l-Thyroxine—*l*-Thyroxine was isolated from hog thyroids as follows: Approximately 9 kilos of fresh thyroid were minced, suspended in 20 liters of water, and heated to 90° by steam. A large amount of fat rose to the surface and was skimmed off after cooling. To the suspension of coagulated thyroid substance was added a solution of 100 gm. of Merck's pepsin, and, after being adjusted to pH 1.5 with hydrochloric acid, the whole was incubated

at 38° for 3 days, pH 1.5 being maintained by the addition of hydrochloric acid from time to time. The digest was then adjusted to pH 5 with sodium hydroxide and filtered by gravity through coarse fluted filters.¹ Filtrate A was saved.

The material insoluble at pH 5 was washed with warm alcohol which removed most of the fatty material. The products so obtained from two such peptic digests of 9 kilos each were combined, suspended in 6 liters of water, mixed with 50 gm. of Fairchild's trypsin, adjusted to pH 7.5 with sodium carbonate, and incubated at 38° for 30 hours, being kept at pH 7.5. The digest was acidified to pH 5 with acetic acid; the precipitate was collected by filtration and washed with water.

The fraction insoluble at pH 5 was suspended in 1200 cc. of water and digested 24 hours at 38° and pH 7.5 with a glycerol extract of pig intestinal mucosa.² The digest was acidified to pH 5 with acetic acid, and after about 20 hours the precipitate was collected by centrifugation.

To complete the hydrolysis the acid-insoluble fraction was refluxed 10 hours with 20 times its weight of 2 *N* sulfuric acid. The hydrolysate was adjusted to pH 5; the dark colored precipitate was collected and washed with water on the centrifuge, and dissolved in 2 liters of water with the help of a few cc. of ammonium hydroxide. The solution was warmed to 60° and treated with a slight excess of warm barium hydroxide solution, which caused precipitation of most of the dark colored impurities. The solution was filtered quickly and the pale yellow filtrate acidified to pH 5 with acetic acid, which caused the separation of a light cream-colored precipitate. The insoluble barium compounds from the foregoing treatment were suspended in water, treated with a few cc. of ammonium hydroxide, and stirred with a slight excess of sodium sulfate. The barium sulfate was filtered off; the filtrate was diluted to 2 liters, again treated with a slight excess of barium hydroxide, filtered quickly, and acidified to pH 5, when a small second crop of insoluble substance was obtained. The two acid-insoluble precipitates were combined and washed with water.

¹ The filtration was troublesome because of the large amount of fat still present. It would undoubtedly have been better to have started the digestion with defatted material.

² Kindly given to us by Professor Max Bergmann.

Further purification was effected (as was done by Harington (3)) by crystallization of the monosodium salt, for which purpose the material was warmed with 0.5 per cent sodium carbonate solution, the insoluble impurities were separated by centrifuging, and on cooling in the ice box the salt of thyroxine separated in crystalline form. Crystallization in this manner was repeated (three times) until the substance was pure white, whereupon it was dissolved in 80 per cent alcohol containing sodium hydroxide and acidified with acetic acid, which caused the separation of the free acid in pure form.

The yield of pure thyroxine was 104 mg., only a small fraction of the total amount of thyroxine one would expect to be present in 18 kilos (wet weight) of average hog glands.

The polariscopic examination was made with a high grade Schmidt and Haensch half shadow polarimeter; the light source was an electric sodium vapor lamp.

100 mg. of substance dissolved in 3 cc. of a solution composed of 24 gm. of 0.5 N sodium hydroxide and 56 gm. of absolute alcohol; tube length, 1 dm.

Ten readings on the solution yielded a mean value of -0.178° , with an average deviation of $\pm 0.013^\circ$. Six readings with an empty tube gave a mean of -0.031° , with an average deviation of $\pm 0.005^\circ$. Hence the observed rotation was -0.147° ; whence $[\alpha]_D = -4.4^\circ$.

The specific rotation of our material is slightly greater than that observed by Harington and Salter (4) who reported -3.8° both for the material obtained by resolution of synthetic thyroxine and for thyroxine isolated from the thyroid gland. The difference between our specific rotation and that of Harington and his collaborators may be even slightly greater, because Harington used the mercury green light, which commonly gives somewhat higher values than does sodium light.

Whether the difference between our rotation and that of Harington is significant is uncertain, because the observed rotations were small and the errors consequently large.

The substance was recovered after polariscopic examination and analyzed for iodine and nitrogen.

<i>Analysis</i> — $C_{15}H_{11}O_4NI_4$.	Calculated.	N 1.80, I 65.4
	Found.	" 1.82, " 65.1, 65.0

This specimen was used for the bioassays reported here.

A second preparation obtained in the same manner, except that the starting material was 2.2 kilos of commercial desiccated and defatted thyroid powder, yielded 28 mg. of analytically pure thyroxine. The mean observed rotation was -0.055° for a 0.93 per cent solution in a 1 dm. tube.³ Analysis showed 65.0 per cent iodine.

Note on Thyroxine Content of Acid-Soluble Fraction after Peptic Digestion

It has been assumed by some investigators (cf. Salter and Pearson (5)) that the acid-soluble fraction after peptic digestion of thyroid protein is free from thyroxine. This is far from being the case. Very large losses of thyroxine resulted in the isolation described above from discarding the acid-soluble fractions after each step in the hydrolysis. Filtrates A from the two peptic digests described above contained 2.64 and 2.70 gm. of total iodine (approximately half of the total iodine in the thyroid material used). Of this 0.170 and 0.155 gm. (6.4 and 5.7 per cent respectively) were present as thyroxine as determined by the method of Leland and Foster.

Some experiments performed on one of these peptic digest filtrates are of interest in connection with the recent paper of Salter and Pearson (5), and are briefly reported here.

A portion of Filtrate A which contained 5.7 per cent of its iodine in the form of thyroxine was boiled for 5 minutes to inactivate any remaining pepsin and was then concentrated under reduced pressure to one-tenth of its volume. An aliquot of the concentrate was mixed with pepsin and incubated at pH 1.5 for 24 hours. It was then diluted with water to its original volume and adjusted to pH 5 with sodium hydroxide. A precipitate separated which when washed and dried was found to contain 0.284 per cent total iodine, of which 15 per cent was in the form of thyroxine. This confirms the *observation* of Salter and Pearson. However, we cannot accept the interpretation of these investigators, who neglected the presence of thyroxine in their filtrates and suggested that the

³ The corresponding specific rotation is -5.9° , but, since the observed rotation is so small, the measurement has scarcely more than qualitative significance.

pepsin added to the concentrates caused the synthesis of thyroxine or some closely related (and presumably, active) substance. We have found that a similar partially selective precipitation of the thyroxine-containing peptides is obtained in the absence of pepsin when egg albumin is used in its place. The denatured egg albumin precipitates at pH 5 and apparently carries down somewhat selectively the thyroxine-containing peptides. The precipitate contained 0.20 per cent total iodine, of which 18.5 per cent was in the form of thyroxine.

Pepsin which had been boiled 5 minutes gave a product having 0.16 per cent total iodine, of which 15.5 per cent was as thyroxine.

The peptic digest filtrate obtained at pH 5 if acidified more strongly (to about pH 3) yields a precipitate of protein material with relatively high phosphorus content (2.6 per cent), which probably represents a salt of peptone and nucleic acid. This precipitate was found to have 0.42 per cent total iodine with 21 per cent of it as thyroxine. It appears, therefore, that a protein precipitate forming in such a solution carries down with it somewhat selectively the thyroxine-containing peptides which obviously are not completely removed from a peptic digest at pH 5.

No attempt was made to isolate thyroxine from the acid-soluble products of peptic digestion. However, in the second preparation of thyroxine, from the commercial thyroid powder, the material soluble at pH 5 after *tryptic* digestion yielded 33 mg. of pure *l*-thyroxine after the hydrolysis was completed by boiling with acid, clearing with barium hydroxide, and purifying through the monosodium salt, as described above. This is actually more than was obtained from what was regarded as the main thyroxine fraction. However, yields in these preparations have no quantitative significance.

Biological Assays—The biological assays were based on the measurement of oxygen consumption of guinea pigs according to the method of Krogh and Lindberg (6).

Two groups of seven guinea pigs each received subcutaneously the sodium salt of *l*-thyroxine every other day over a period of 14 days. Daily determinations of the increase over the normal oxygen consumption were made. Details of the routine have been described previously (1).

Great care was taken to prevent the racemization of the *l*-

TABLE I

Effects of Administration of l-Thyroxine, dl-Thyroxine, and Thyroid Gland to Guinea Pigs

Preparation	Iodine content	Dose administered*	Increase in O ₂ consumption		Average weight loss on 14th day of dosage period
			Mean of last 8 days of individual animals	Mean of group	
	<i>per cent</i>	<i>mg. I per sq. m.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent average initial body weight</i>
<i>l</i> -Thyroxine, Group 1, 7 animals	65.1	0.262 as disodium salt injected subcutaneously	50.1	40.3	5.6
			38.8		
			51.4		
			36.1		
			45.2		
			31.4		
			29.3		
<i>l</i> -Thyroxine, Group 2, 7 animals	65.1	“ “	39.5	37.6	9.3
			37.4		
			37.5		
			38.6		
			27.5		
			47.3		
			35.4		
<i>dl</i> -Thyroxine, 6 animals	64.9	“ “	24.5	23.1	3.7
			26.6		
			19.9		
			23.4		
			25.7		
			18.4		
			35.8		
Burroughs Wellcome commercial thyroid preparation as standard, 6 animals	0.254 (Total iodine)	1.13 total I	28.7	35.3	6.6
			34.0		
			39.3		
			38.6		
			35.1		
			23.2		
			8.6		
<i>dl</i> -Thyroxine, 6 animals	63.3	0.262 as disodium salt by mouth	21.3	17.9	0.0
			21.1		
			14.8		
			18.4		

* Administered every other day for 14 days.

† As determined by the method of Leland and Foster.

thyroxine. Samples of approximately 1.5 mg. were weighed into sterile beakers, dissolved in 4 drops of sterile 0.1 N sodium hydroxide, and made up to volume with sterile distilled water. The standard dose, 0.262 mg. of thyroxine iodine per sq. m., in a volume of approximately 1 cc. was injected immediately without heating. Fresh solutions were prepared for each of the seven injections received by the animals.

For an exact comparison with *l*-thyroxine, equal doses of pure racemic thyroxine were administered in an identical manner to a group of six guinea pigs.

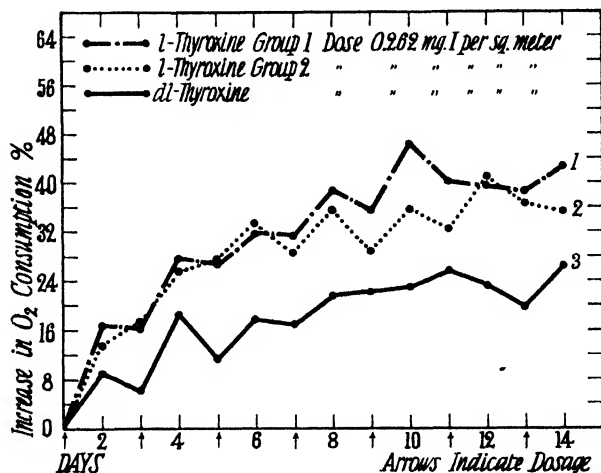


FIG. 1. Comparison of the increases in oxygen consumption produced by *l*-thyroxine and by *dl*-thyroxine.

The results of these experiments are shown in Table I and Fig. 1.

Composite Curves 1 and 2 of Fig. 1 show the results obtained with *l*-thyroxine. Each point was determined by averaging the increase in oxygen consumption over the normal of the seven animals of the group. For comparison of different curves a mean value was obtained by averaging the last 3 days of the 14 day period. This was regarded as the full effect of the dose. As shown in Table I, the mean value of the last 3 days of Group 1 of animals receiving *l*-thyroxine was 40.3 per cent, while that of Group 2 was 37.6 per cent, which is within the limit of error of this method.

The results obtained when racemic thyroxine was injected under identical conditions (Fig. 1, Curve 3) are markedly lower over the entire 14 day period. The mean of the last 3 days of the group was only 23.1 per cent as compared with 37.6 and 40.3 per cent obtained with *l*-thyroxine. From this it appears that the *l* form is much more active than the *d* form.

For comparison of our assays of *l*-thyroxine with our previous studies (1) of racemic thyroxine and of thyroid gland, some data previously published are presented again in Fig. 2 and in Table I.

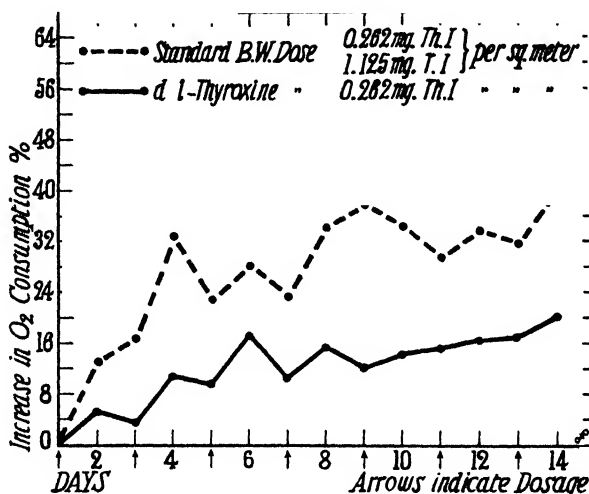


FIG. 2. Comparison of the increases in oxygen consumption produced by the standard thyroid preparation (Burroughs Wellcome) and by *dl*-thyroxine. *Th.I* = thyroxine iodine; *T.I* = total iodine.

Two composite curves are shown in Fig. 2, each curve the mean of a group of six animals. The upper curve shows the effect observed when Burroughs Wellcome thyroid powder, used as a standard, was administered by mouth. A 35.3 per cent increase over the normal oxygen consumption was found when the results of the last 3 days were averaged. The results shown in the lower curve (Fig. 2) were obtained when an amount of the sodium salt of racemic thyroxine equivalent to the thyroxine content of the standard (as determined by the method of Leland and Foster) was administered in a similar manner. The mean of the last 3 days of the group in

this case was only 17.9 per cent. It should be noted (Table I) that one of the six animals included in the group was relatively insensitive to the dose, which tended to lower the group mean. The average of the remaining five animals was 19.8 per cent. Approximately 50 per cent only of the increase in oxygen consumption which was obtained with the standard thyroid powder was observed with racemic thyroxine. Subcutaneous injection of the same dose over a second 14 day period failed to raise the figure significantly.

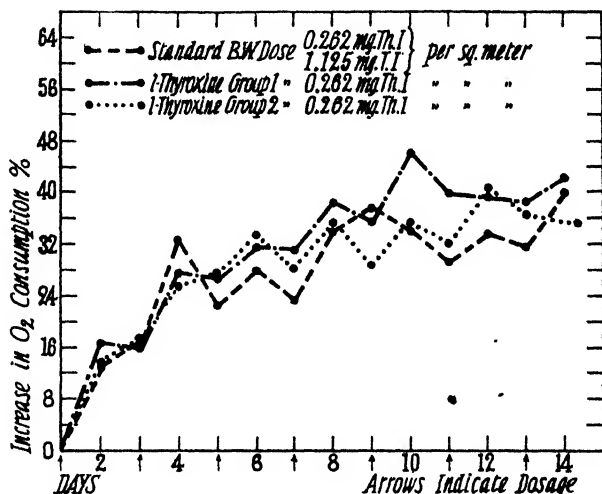


FIG. 3. Comparison of the increases in oxygen consumption produced by the standard thyroid preparation (Burroughs Wellcome) and by *l*-thyroxine. Th.I = thyroxine iodine; T.I = total iodine.

Fig. 3 shows the two *l*-thyroxine curves together with that of the standard thyroid substance of Fig. 2 in equi-thyroxine doses. Throughout the entire 14 day period the three curves follow one another so closely that the response must be considered the same for all three within the limits of error of the method. The conclusion may safely be drawn that the calorogenic activity of pure thyroxine is fully equivalent to that of an equi-thyroxine dose of thyroid gland, provided the thyroxine is administered as the naturally occurring isomer.

DISCUSSION

Our data permit some comment on the question of the relative activities of *d*- and *l*-thyroxine. As shown in Fig. 3, the calorogenic activity of a certain parenteral dose of *l*-thyroxine (0.262 mg. of thyroxine iodine per sq. m. every 2nd day) is fully equal to that of the same amount of thyroxine in the form of thyroid substance given by mouth. Exactly the same dose of *dl*-thyroxine (Curve 3, Fig. 1, this paper) had the same calorogenic effect as was reported previously ((1) Table I) for one-half that dosage (*i.e.*, 0.131 mg. per sq. m. every 2nd day) of thyroxine iodine as thyroid. The mean increases of oxygen consumption above the normal periods were 23 per cent for the dosage of 0.262 mg. of thyroxine iodine as *dl*-thyroxine and 22 per cent for 0.131 mg. of thyroxine iodine as thyroid substance ((1) Table I). It thus appears that the calorogenic activity of *dl*-thyroxine is ascribable, within the rather wide limits of accuracy of our method, solely to the *l* component. The observation by Gaddum (7) of some activity in *d*-thyroxine may perhaps be attributed to incomplete resolution in his sample, which possessed an appreciably lower specific rotation than that of the natural product used in our experiments.

Salter, Lerman, and Means (8) administering the same preparations as used by Gaddum to myxedema patients failed to find any difference between the two forms.

SUMMARY

1. *l*-Thyroxine ($[\alpha]_D = -4.4^\circ$) was isolated from thyroid glands and its calorogenic activity compared with that of pure *dl*-thyroxine and equi-thyroxine doses of thyroid substance.

2. *l*-Thyroxine was found to have a calorogenic effect on normal guinea pigs approximately twice as great as that of racemic thyroxine.

3. The calorogenic activity of a certain dosage of thyroid gland is quantitatively accounted for by the *l*-thyroxine which it contains.

4. The acid-soluble fraction of a peptic digest of thyroid protein contains an appreciable amount of thyroxine, presumably still in peptide linkage. From such a solution, the thyroxine-containing peptides are somewhat selectively carried down on various protein precipitates which may be caused to form in the solution.

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THE ULTRAVIOLET ABSORPTION SPECTRUM CURVE OF PHTHIOL, A PIGMENT OF THE HUMAN TUBERCLE BACILLUS

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PLATE 1

(Received for publication, June 18, 1936)

Phthiol is a pigment which Anderson and Newman (1) have isolated in the form of yellow prismatic crystals from the acetone-soluble fat of the human tubercle bacillus, Strain H-37, grown on the Long synthetic medium. The composition of the pigment corresponds to the formula $C_{11}H_8O_3$ and it has been identified by Anderson and Newman (2) as 2-methyl-3-hydroxy-1,4-naphthoquinone. Since the pigment is present in very small amounts in the bacillus, a method was developed by which it could be produced synthetically for experimental study; the synthetic product is identical with the natural phthiol (3). A study of the physical characteristics of the pigment is of interest. Dhéré (4) has already made a study of the fluorescence; this paper presents the ultraviolet absorption spectrum curve, in the range λ 4550 to λ 2160 Å., of a sample of the synthetic phthiol which Dr. Anderson very kindly supplied. The crystals of synthetic phthiol in ordinary white light and in polarized light are illustrated in Text-fig. 1.

The quantitative ultraviolet absorption spectrum curve is shown in Text-fig. 2. The logarithm of the molecular extinction coefficient $\epsilon = (1/cd) \log (I_0/I)$, where c is the concentration, d the depth of the cell in cm., I_0 the intensity of the incident light, and I the intensity of the emergent beam, is plotted against the wavelength in Ångström units. The wave numbers in cm.^{-1} are also indicated on the abscissa.

The regions where selective absorption is indicated are a shallow

band, between λ 4000 and 3700 \AA ., with the maximum at about λ 3850 \AA . where $\log \epsilon$ is 3.15; a band at about λ 3340 \AA ., $\log \epsilon = 3.44$, minimum at λ 3040 \AA ., $\log \epsilon$ 3.15; and two sharp bands in the λ 3000 to 2200 \AA . region with maxima at about λ 2780 \AA ., $\log \epsilon$ 4.21, and at λ 2500 \AA ., $\log \epsilon$ 4.31, and with minima at λ 2650 and 2270 \AA ., where $\log \epsilon$ values are 4.00 and 3.84 respectively.

A series of qualitative spectrograms (Figs. 1 and 2) were made with a Hilger No. E-316 quartz spectrograph by varying the cell depths and the concentrations to indicate the regions of selective

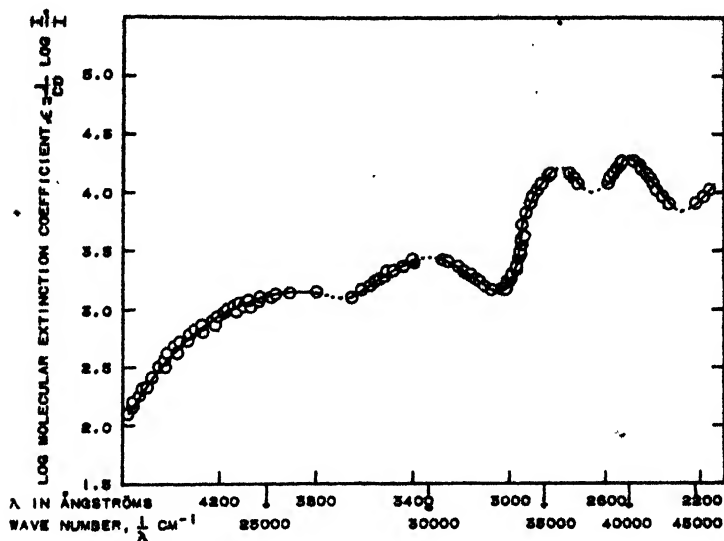


TEXT-FIG. 1. Photomicrographs of crystals of synthetic phthiocol. $\times 60$. The left-hand section shows crystals photographed in ordinary white light; the other two photographs in polarized light.

absorption near λ 3850, 3340, 2780, and 2500 \AA . The solvent used was 95 per cent alcohol.

In an attempt to determine the rôle played by the different parts of the phthiocol molecule in its ultraviolet absorption, consideration was given to 1,4-naphthoquinone and to substances which differ from phthiocol only in that the methyl and the hydroxyl groups are omitted. The ultraviolet absorption was determined for a 95 per cent alcoholic solution of 1,4-naphthoquinone (Eastman), the only one of these substances available. The $\log \epsilon$ values are indicated by the crosses in Text-fig. 3 in which the curve for 1,4-naphthoquinone is plotted with that for phthiocol for comparison.

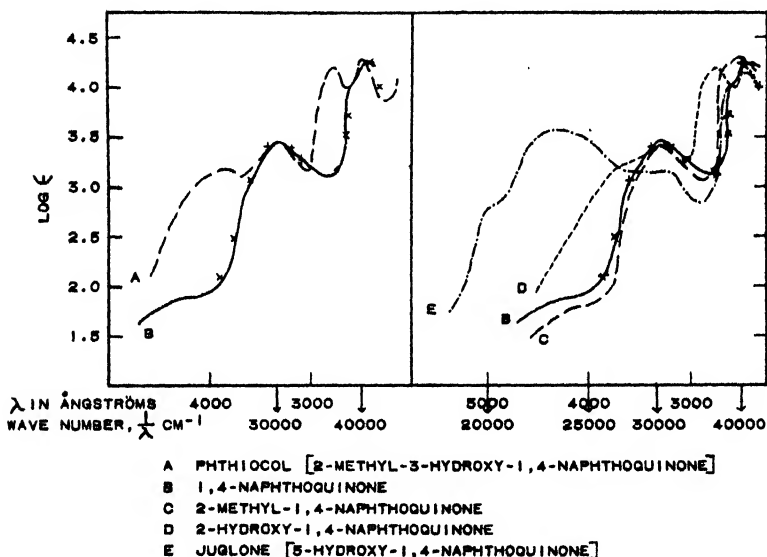
The maximum for the band at λ 3340 Å. occurs at the same wavelength as one of the bands in the curve for phthiocol and the $\log \epsilon$ values at this wave-length are the same, 3.44 for both substances. Therefore, the absorbing entity responsible for this band is present in both 1,4-naphthoquinone and phthiocol. The maximum of the band is not displaced nor is the value of the molecular extinction coefficient at this point changed by the substitution of hydroxyl and methyl groups in the 1,4-naphthoquinone, in positions (2) and (3) respectively.



TEXT-FIG. 2. The ultraviolet absorption spectrum curve of synthetic phthiocol in 95 per cent alcohol.

Morton, Hassan, and Calloway (5), in a study of the absorption spectra in relation to the constitution of keto-enols, have shown that there are three bands in the ultraviolet range which are associated with the C_6H_5CO group. One of these, of low intensity, occurs near 310 to 320 $m\mu$ and is due to the carbonyl group, $C=O$, while that at λ 280 $m\mu$ is due to the $C=O$ group influenced by the phenyl group, $C_6H_5-C=O$. That at λ 242 $m\mu$ is due to the phenyl group influenced by carbonyl; that is, $C_6H_5-C=O$.

The selective absorption noted in the curves for phthiocol in Text-fig. 3 and for other naphthoquinones occurs close to their ranges. A probable correlation between the bands for the naphthoquinones and the maxima observed by Morton, Hassan, and Calloway to be characteristic of the C_6H_5CO group has been suggested by Macbeth, Price, and Winzor (6). It is possible that the selective absorption observed for phthiocol is due to the same absorbing mechanism which is present in the naphthoquinone but modified by other portions of the phthiocol molecule.



TEXT-FIG. 3. Ultraviolet absorption spectrum curves

The absorption observed in the λ 3340 Å. region for phthiocol and for 1,4-naphthoquinone occurs in many of the spectra of the quinones. Morton and his colleagues have pointed out that absorption bands in the ranges λ 320 to 308, 290 to 280, 250 to 240 $m\mu$ are observed in many compounds containing the $C:C-C:O$ group but not the phenyl group; hence, the absorption must be in either a $C:C$ or a $C:O$ group. If the electron which is raised to a higher energy level forms part of the $C:C$ linkage, the molecular extinction coefficients are generally high, of the order of 10^3 to

10⁵, as they are in the case of the naphthoquinones in the λ 330 $m\mu$ region. Therefore it is probable that the absorption in this region is due to an absorption by the C:C linkage.

After the work reported in this paper was undertaken, it was found that Macbeth, Price, and Winzor (6), in connection with a study of the coloring matters of the insectivorous plant *Drosera whittakeri*, droserone and hydroxydroserone, had investigated the spectra of representative hydroxynaphthoquinones. The curves which are reproduced in their paper for 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, and 5-hydroxy-1,4-naphthoquinone (juglone) are designated in Text-fig. 3 as Curves B, C, D, and E respectively. The crosses represent $\log \epsilon$ values obtained in this laboratory for 1,4-naphthoquinone (Eastman). The general similarity in Curves B and C and the differences between Curve B and Curves D and E are apparent.

The spectra investigated by Macbeth, Price, and Winzor show that the "introduction of hydroxyl groups in most cases modifies the absorption of 1:4-naphthaquinone and 2-methyl-1:4-naphthaquinone which may be regarded as typical of the 1:4-structure." A substituted methyl group in position (2) does not greatly alter the general contour of the absorption curve for 1,4-naphthoquinone except in the λ 2800 to 2400 Å. region.

SUMMARY

The ultraviolet absorption curve for synthetic phthiocol is recorded. Consideration of this curve indicates that the same absorbing mechanism which is present in the naphthoquinones may be responsible for the selective absorption observed but modified by other portions of the phthiocol molecule—methyl groups in the λ 2800 to 2400 Å. region, and the hydroxyl groups.

I wish to thank Miss Amy Walker for valuable technical assistance throughout this study.

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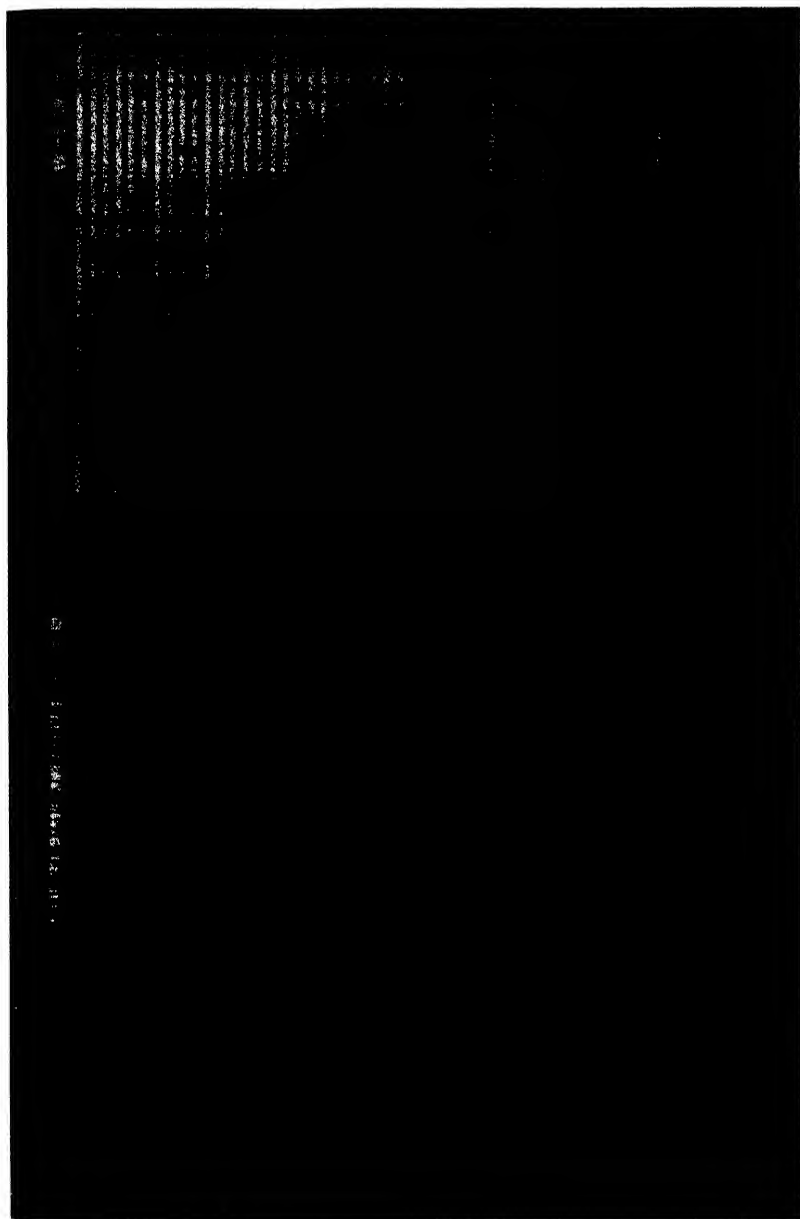
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EXPLANATION OF PLATE 1

FIG. 1. Ultraviolet absorption spectra of phthiocol at concentrations 0.0001878 M and 0.00093905 M. Cell depth, 2.497 to 79.52 mm.

FIG. 2. Ultraviolet absorption spectra of 1,4-naphthoquinone at concentrations 0.0002404 M and 0.001202 M. Cell depth 2.497 to 79.52 mm.



(Crowe: Absorption spectrum of phthiocol)

THE EFFECT OF ALTITUDE ON THE AFFINITY OF HEMOGLOBIN FOR OXYGEN*

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(Received for publication, May 23, 1936)

When the human organism is subjected to a diminished partial pressure of oxygen, it rapidly begins to make various adjustments to its new environment. One of the most certain of these is an increase in the concentration of hemoglobin in the blood. This is probably attributable to various adjustments, some of which are of a temporary or emergency nature and others of a more permanent sort which persist as long as the individual remains under diminished oxygen pressure. Barcroft (1925) points out that the temporary adjustments probably include the abstraction of water from the blood, the contraction of the spleen, and the opening of capillary areas, while the more permanent sort includes an increased activity of a new equilibrium between the production and destruction of red blood cells.

Of what value is this increase in hemoglobin concentration to an organism living in high altitudes? The first answer that comes to mind is that the oxygen reserve in the blood is increased. Another thought lurks in the background—perhaps the new hemoglobin has a property different from the old. Perhaps its affinity for oxygen is greater, enabling the blood of the organism to leave the lungs with a greater oxygen saturation.

The idea is not illogical, when consideration is given to numer-

* The International High Altitude Expedition was supported in part by grants from: the Fatigue Laboratory, Harvard University, the Milton Fund for Research, Harvard University, Duke University, Columbia University, the National Research Council, the Royal Society (London), the Rask-Ørsted Fund (Copenhagen), the Corn Industries Research Foundation, American Association for the Advancement of Science, the Josiah Macy, Jr., Foundation, and the various university departments concerned

ous observations made to show the variability of hemoglobin. Hemoglobin produced in the fetus seems to have an affinity for oxygen unlike that of the blood of its mother (Barcroft *et al.*, 1935). This suggests a relationship between the partial pressure of oxygen and the affinity of hemoglobin for oxygen—the fetal hemoglobin, which is habitually exposed to a lower O₂ pressure than the maternal, having a higher affinity. Brinkman and Jonxis (1935) have also shown that two hemoglobins probably exist simultaneously in the blood of adult human beings. In the developing chick where there is a smaller supply of oxygen, hemoglobin possesses a higher affinity for oxygen than does hemoglobin of the adult fowl (Hall, 1935). McCarthy (1933) has also shown that fetal and maternal hemoglobins differ.

Consequently, it seemed desirable to make a study of the affinity of hemoglobin for oxygen in men who were going from low to high altitudes. There are three ways that such a problem might be studied: (1) determination of the oxygen dissociation curve of whole blood as drawn from the subject by equilibrating such blood with approximate gas mixtures (this has been done and has been reported elsewhere (Keys, Hall, and Barron, 1936)); (2) purification and recrystallization of hemoglobin and subsequent study of its properties (such a procedure would be extremely difficult if not impossible on a field expedition); (3) hemolysis of blood and dilution with appropriate buffers followed by spectroscopic examination for affinity of hemoglobin for oxygen.

The latter method has been found useful in other problems (Hall, 1934, 1935) and it is adaptable for work in the field. Its advantages are speed, a precise control of salt and hydrogen ion concentration, and portability of the apparatus. There are limitations; low and high saturation are not as accurately determined as with the first two methods and the hemoglobin is not in its natural physiological environment.

Analyses were made as previously described (Hall, 1935), but the apparatus was modified somewhat for field work. Flash-light batteries and lights were used for illumination and spring phonograph motors for rotating tonometers and stirring the bath. Alcohol lamps were used for heating the bath. The temperature of the bath was maintained at $37.0^{\circ} \pm 0.1^{\circ}$. All dissociation curves reported in this paper were made on hemoglobin in $M/15$ phosphate buffer solutions at pH 6.8.

TABLE I
Stations Occupied, Pressure, and Elevations Recorded

Station	Period of occupancy	Corrected barometric pressure	Altitude	
		mm. Hg	km.	ft.
Chuquicamata	Apr. 8-June 4	543	2.81	9,200
Ollague	June 5-13; June 25-July 18	489	3.66	12,020
Montt	June 13-25	429	4.70	15,440
Aucanquilcha	June 26-July 15	401	5.34	17,500
Punta de Cerro	June 29-July 14	356	6.14	20,140

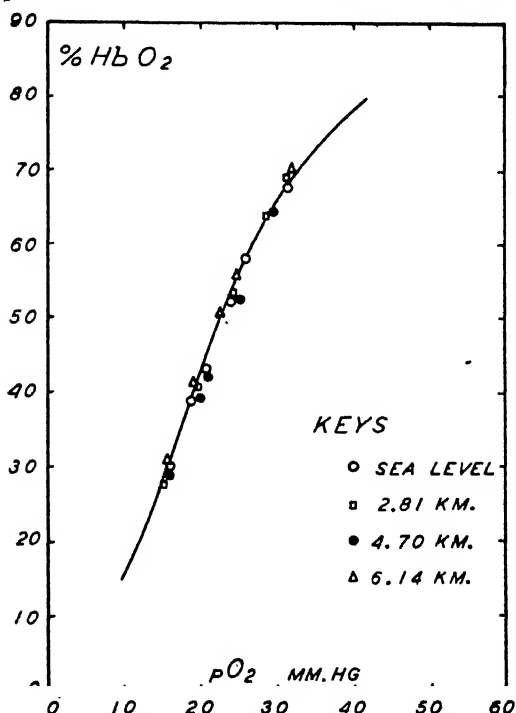


FIG. 1. The oxygen dissociation curve of hemoglobin (Keys) at different altitudes (37.0° and pH 6.8).

There were ten members of the party of the International High Altitude Expedition. Samples of blood were taken for these experiments from all save one. A few analyses were made on

blood of residents who were living at 17,500 feet. The itinerary and general description of the expedition are given elsewhere (Keys, 1936). Stations occupied, pressure, and elevations recorded are listed in Table I. Blood samples were drawn from subjects near the end of their stay at various altitudes. In the case of Keys at Punta de Cerro blood was drawn on the 6th day and in the case of F. G. H. only 2 hours after arrival at the summit.

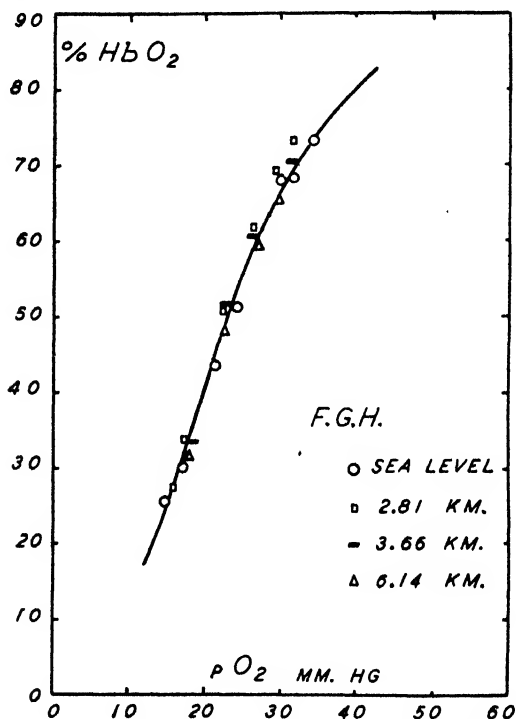


FIG. 2. The oxygen dissociation curve of hemoglobin (F. G. H.) at different altitudes (37.0° and pH 6.8).

Time did not permit determination of dissociation curves on every member of the expedition at every station. More analyses were made on the blood of Keys than on any other member. Results of analyses on his blood at various altitudes are shown in Fig. 1 and it will be observed that no significant shift in the dissociation curve occurred. A similar set of experiments was per-

TABLE II

Showing Position of Oxygen Dissociation Curve of Members of Expedition and Native Miners at Various Altitudes

Hemoglobin was equilibrated in a phosphate buffer solution (M/15) at pH 6.8 and 37.0° and percentage saturation read by the spectroscopic method. Partial pressures of oxygen required for half saturation are given.

	Subject	Altitude	Oxygen capacity	pO_2 for Hb = HbO ₂
		km.	vol. per cent	mm. Hg
Expedition members	Barron	2.81	22.6	22.0
	Christensen	0	18.2	23.8
		2.81	21.4	24.0
	Dill	0	18.2	23.0
		2.81	18.4	23.0
		4.71	22.1	24.3
	Edwards	0	21.3	25.2
		2.81	22.7	25.3
	Forbes	0	20.4	24.6
		2.81	22.8	24.0
		6.14	24.56	23.5
	Hall	0	22.0	23.5
		2.81	23.6	22.0
		3.66	24.6	21.0
		5.34	25.9	23.0
		6.14	25.5	22.5
	Keys	0	18.6	23.0
		2.81	19.6	23.0
		4.71	23.1	25.0
		6.14	23.7	22.5
Resident miners	McFarland	2.81	20.8	25.0
	Talbott	0	21.6	23.2
		2.81	22.2	24.0
	Alcaino	5.34	29.8	27.0
	Alcio	5.34	29.3	25.3
	Heredia	5.34	34.2	25.5

TABLE III

Comparison of Oxygen Dissociation Curves of Dilute Solutions of Hb Obtained from Members of Expedition with Those of Residents

	Altitude	pO_2 at HbO ₂ = Hb
	km.	mm. Hg
Party	5.34	23.5
Residents	5.34	26.0

formed on F. G. H. with results as shown in Fig. 2. No significant shift in the position of the curve occurred in this case either.

A summary of all twenty-six curves is given in Table II. The positions of the curves are indicated by the partial pressure of oxygen required to produce half saturation of the hemoglobin (*i.e.*, $Hb = HbO_2$). Apparently, acclimatization to high altitude has little or no effect on the position of the oxygen dissociation curve of a dilute solution of hemoglobin in phosphate buffer at pH 6.8.

The results with the dilute solutions of hemoglobin are not entirely in harmony with those obtained by the direct analytical method on whole blood at constant pH_c (Keys, Hall, and Barron, 1936). In whole blood, the oxygen affinity of the hemoglobin at constant pH_c decreased steadily at all stations above 9200 feet.

When one compares oxygen dissociation curves of dilute solutions of hemoglobin obtained from members of the expedition at high altitude with those of the residents (Table III), there appears to be a small but not insignificant difference. Hemoglobin from residents seems to have less affinity for oxygen than that of sojourners. Unfortunately no observations were made on high altitude residents at sea level or on men of the same racial stock at sea level. Accordingly, there is a possibility that these differences are either racial or individual peculiarities.

SUMMARY

No significant change in the affinity of hemoglobin for oxygen was observed in blood of nine men with change in altitude from sea level to 20,000 feet. Hemoglobin produced in the body during acclimatization to altitude does not appear to differ from that produced at sea level.

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LIVER PROTEINS

I. THE QUESTION OF PROTEIN STORAGE*

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(Received for publication, May 16, 1936)

In the midst of his long and classical paper on glycogen Pflüger (2) remarked that observation of the livers of animals maintained upon high protein diets convinced him that the liver is an organ for the storage of protein as well as of glycogen and fat. No experiments were cited, but it appears that Pflüger's conclusion rested upon cursory observations of the excised liver and its rate of disintegration in hot potassium hydroxide. According to Seitz (3), a student of Pflüger, no convincing experiments had ever been reported and it remained for Seitz to undertake the first satisfactory investigation of the problem. He succeeded in showing that the maintenance of chickens and ducks upon high protein diets did indeed result in a marked enlargement of the liver and a substantial increase in its nitrogen content. Even more convincing was the work of Tichmeneff (4) upon mice. Not content with nitrogen determinations alone, he showed that the proteins *per se* (tannic acid-precipitable fraction) were greatly elevated.

It is evident that these observations, supported by older histological studies such as those of Afanassiew (5), were completely in harmony with the Pflüger-Voit theory of reserve or labile protein and indicated that the liver might properly be regarded as an important depot for such material. Without an attempt to refer to many pertinent studies on nitrogen balance and changes in liver weight, the crucial problem which presents itself can be stated at once. Do the changes in liver size and protein content, associated with differing levels of protein intake, denote the exist-

* A preliminary report of this investigation appeared in the Proceedings of the Fifteenth International Physiological Congress (1).

ence of a fraction which differs chemically from the basal structural proteins of the organ—the “organized,” “tissue,” or “stable” protein of the early investigators? Or do these terms have a metabolic significance only? Is the deposition of liver protein under favorable metabolic conditions the result of a true hyperplasia or hypertrophy in which all the proteins of the organ participate alike?

The purpose of the present paper is to report the results of a number of experiments designed to answer these questions. It can be said in advance that the evidence obtained under the conditions prevailing in this investigation demonstrates that all of the liver proteins participate in the function of storage. No single protein or fraction of the hepatic proteins can be regarded as reserve material in the sense of being chemically distinct from the basal structural proteins of the organ.

EXPERIMENTAL

Groups of rats were maintained for periods of 2 weeks upon diets which differed widely in protein content. The animals were then killed, the livers isolated, and the various fractions of proteins determined quantitatively. We considered at the outset that the deposition of a reserve fraction different in character from the basal structural proteins, which alone would be present in depleted livers, would be reflected by a disproportionately great increase in one of the fractions as we passed from the livers of low protein content of one group to those of high protein content of another. Conversely, if all of the hepatic proteins participate alike in the function of storage, and protein enrichment is the result, solely, of hyperplasia or hypertrophy, all of the fractions isolated in the course of analysis should increase by the same proportion.

The experimental procedures will now be considered in detail.

Diets and Animals—The animals used were white rats of both sexes, varying in age from 4½ to 18 months, and maintained, until selected for these experiments, on an ordinary stock diet.¹

To secure sufficient liver for analysis, three animals were employed at a time in the low protein experiments and two or three in the high protein experiments. The two or three animals so

¹ Cracked wheat 25, oatmeal 25, corn-meal 25, flaxseed meal 10, dried whole milk (Klim) 5, alfalfa meal 5, yeast 5, bone meal 1.5, sodium chloride 0.5, cod liver oil—with lettuce three times per week.

selected were always of the same sex, and were either litter mates or closely similar to each other in age and weight. Each group of two or three was maintained for 2 weeks on a diet of either high or low protein content. The former consisted of casein 85 per cent, yeast powder 5 per cent, alfalfa meal 5 per cent, salt mixture² 5 per cent; the latter of wheat starch and sucrose 85 per cent, yeast powder 5 per cent, alfalfa meal 5 per cent, salt mixture² 5 per cent. A few drops of cod liver oil were given to each rat daily. Each of the forty-five animals on the high protein diet consumed,³ on the average, 11.6 gm. of the ration per day and increased in weight during the 2 week period from an average of 254 and 198 gm. initially for males and females, respectively, to 263 and 209 gm., respectively, at the time of killing. The corresponding figures for 62 animals on the low protein diets are 13.6 gm. of food per day, initial weights of 274 and 200 gm. for males and females, respectively, and final weights of 232 and 177 gm., respectively. The loss of weight on the low protein diet is, of course, the direct consequence of protein starvation which the diet was designed to accomplish.

Removal of Liver—Under ether anesthesia each animal was bled as quickly and completely as possible from the dorsal aorta by use of syringe and needle. In the experiments of Part C the vena cava was then cut and a moment later, when the liver was judged to be as free from blood as could be obtained without perfusion, the organ was excised and immersed in liquid air.

In the experiments of Parts A and B perfusion with 0.85 per cent sodium chloride (containing potassium phosphate, 0.01 M and of pH 7.4) was employed to free the liver of residual blood. After bleeding from the dorsal aorta, the portal vein was exposed and isolated, the thorax quickly opened, and the vena cava cut above the diaphragm. Approximately 40 cc. of the perfusion fluid, heated to 38°, were then injected into the portal vein and perfused through the liver with very gentle pressure. A broad gage needle was used and more than 3 or 4 minutes never elapsed between the

² Osborne and Mendel's salt mixture was used (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913)).

³ The values for food consumption are drawn from a parallel and strictly comparable experiment on rats in which the food intake, per group of four, was determined daily.

commencement of bleeding and the end of the perfusion. In most cases the perfusion was entirely successful and the liver on excision was uniformly pale brown in color. In possibly one-fifth of the animals the residual blood could not be removed satisfactorily. Blotched areas, sometimes of considerable size, and occupying much of the peripheral portions of the lobes indicated failure of the fluid to penetrate. These livers were invariably rejected. To provide for such a contingency the groups of animals used in most of the low protein perfusion experiments were enlarged from three members to four, permitting an occasional liver to be discarded without occasioning the analytical uncertainties that arose when only two small livers were available for analysis. Although a number of fractionations were made on only 9 or 10 gm. of liver we considered 15 to 25 gm. to be a more desirable quantity.

The livers, on removal, were found to be quite flaccid and gave no evidence of engorgement with perfusion fluid. Each liver was quickly sponged on filter paper, immersed in liquid air, powdered by mortar and pestle, and weighed. The liver powders thus obtained from the three members of a group were combined and the protein fractionation commenced.

Fractionation of Proteins—Since we propose to discuss the problem of fractionation of the liver proteins in another paper, it will be sufficient, for the present purpose, to describe briefly the procedure which was finally adopted and to comment on only a few of the most significant points.

The frozen liver powder was washed into a cylinder⁴ contained in an ice bath with cold 5 per cent sodium chloride. 10 cc. of the salt solution were used for every gm. of liver powder. The suspension was adjusted to approximately pH 5.0 by the addition of dilute acetic acid and use of a spot plate with methyl red or brom-cresol green as an external indicator. It was then placed in a cold room at 0–3° and stirred mechanically for 1 hour. The

⁴ If a large size centrifuge with cups of 250 or 300 cc. capacity is available, the use of such cups instead of a cylinder is recommended. In recent work we have been carrying out six fractionations simultaneously and we find that direct extraction in the centrifuge cups not only saves time but avoids such errors as are due to transfer of material from one piece of apparatus to another.

mixture was centrifuged and the centrifugate containing the salt-soluble material was transferred to an ice bath and kept at 0-3°, while the residue underwent a second extraction. For this purpose the residue was refrozen with liquid air, again powdered, and mixed with cold 5 per cent sodium chloride until the final volume was two-thirds of that which was obtained in the first extraction. The pH was again adjusted to 5.0, the mixture stirred for 1 hour at 0-3°, and then centrifuged. This second centrifugate was combined with the first portion, mixed, and the total volume recorded.

Portions of 5 cc. and 10 cc. were removed for nitrogen determinations. The values so obtained will be referred to as salt-soluble nitrogen.

The remainder of the salt-soluble fraction was transferred to a viscose tube,⁵ covered with a layer of toluene, immersed in cold distilled water in a deep cylinder, and dialyzed for 4 days against running distilled water at 0-3°. The rate of flow was so adjusted that approximately 15 liters of water were used daily.

Meanwhile, the liver residue remaining after the two extractions with sodium chloride was twice extracted with cold 0.25 per cent sodium hydroxide. The volumes of sodium hydroxide used were the same as the volumes of sodium chloride employed in the initial extractions. In each case the mixture was stirred continuously for 1 hour at 0-3° and the centrifugates combined and measured. There was no intermediate freezing with liquid air. Of the combined extract, referred to as the alkali-soluble fraction, 5 and 10 cc. portions were removed for nitrogen determinations. The remainder was adjusted to pH 4.5 to 5.0 by the addition of dilute acetic acid and permitted to stand overnight at 0-3° for complete precipitation of the alkali-soluble protein (Globulin II).

Here it might be mentioned that isolation of the liver, extraction with sodium chloride and sodium hydroxide, commencement of dialysis of the salt-soluble fraction, and precipitation of Globulin II should all be done on the same day. Occasionally, though rarely, we have been obliged to delay the extraction with alkali until the 2nd day. There was no evident change in the proteins contained within the residue as a result of standing overnight but,

⁵ Visking Corporation, Chicago.

having had occasion to observe rather marked proteolytic activity in liver preparations, even at 0°, we endeavored to minimize delay in the initial stages of fractionation.

On the 2nd day the copious precipitate of Globulin II was centrifuged off and the nitrogen content of the centrifugate determined by analysis of an aliquot portion. This fraction we shall refer to as the Globulin II filtrate nitrogen.⁶

The residue, always quite small, which remained after the two alkali extractions was immersed in 1 per cent acetic acid, centrifuged off on the following day, dried out under alcohol by the same procedure which is described for Globulin II,⁶ and weighed. We shall refer to it as the final residue.

In many of the introductory experiments we followed up the alkali extraction with a single extraction with 2 per cent hydrochloric acid but the amount of nitrogenous material that went into solution was so small that it seemed justifiable to abandon the acid extraction.

To return to the salt-soluble fraction, the dialysis was continued for 4 days, although even by the end of the 2nd day inspection of the fraction seemed to suggest that separation of the euglobulin was virtually complete. The contents of the tube were then centrifuged and aliquot portions of the centrifugate were removed for nitrogen determinations. This fraction was designated as the pseudoglobulin-albumin fraction. The euglobulin was redissolved in 5 per cent sodium chloride and redialyzed for 24 hours against running distilled water at 0-3° and then dried under alcohol by the same procedure adopted for the Globulin II. After the final desiccation at 105° the material was cooled and weighed.

⁶ Hitherto in all experiments we have collected, reprecipitated, dried, and weighed the Globulin II fraction. Our object in so doing was to accumulate a sufficient quantity for chemical analysis and also to obtain a gravimetric check upon the values for the Globulin II content as calculated from the nitrogen determinations before and after precipitation. It should be pointed out that direct drying of Globulin II is not possible since it shrinks to a hard dense mass and even after a week at 105° does not attain a constant weight. We have found it convenient to dry out the reprecipitated material, still in the centrifuge tube, by means of 95 per cent alcohol. The mixture is frequently stirred and the alcohol centrifuged off and replaced daily for 4 or 5 days. It is now filtered, washed with alcohol and ether, and dried overnight at 105°. A light fluffy powder is obtained. It dries quickly and is not hygroscopic.

From the pseudoglobulin-albumin fraction, the pseudoglobulin was precipitated by the addition of anhydrous sodium sulfate to a final concentration of 1.5 M. After being maintained for 1 hour at 38° the mixture was filtered and the precipitate washed with a few cc. of 1.5 M sodium sulfate.⁷

From the albumin fraction, obtained on filtering off the salted-out pseudoglobulin, an aliquot portion was removed for a nitrogen determination and the albumin contained within the bulk of the solution was removed by heat coagulation and filtration.⁷ A final nitrogen determination was run upon the albumin-free filtrate.

The procedure outlined above yielded five fractions, Globulin II, euglobulin, pseudoglobulin, albumin, and final residue, of which the first two were the most abundant. At first we were disposed to regard Globulin II as a nucleoprotein⁸ by virtue of its solubility in dilute sodium hydroxide and complete precipitation on acidification. However, it cannot be the same as the highly colored, hygroscopic, preparations of so called nucleoprotein, obtainable in relatively small quantities by acetic acid precipitation of hot water extracts and described as liver nucleoprotein, ferratin, or hepatin by various investigators (Zaleski (6), Schmiedeberg (7), Vay (8), Wohlgemuth (9), Scaffidi (10), Salkowski (11)). Nor does it seem to be quite the same as Halliburton's nuclealbumin (12), of which little was reported present in liver. Its relatively low phosphorus content (0.6 per cent) also distinguishes it from the liver nucleoprotein (0.96 to 1.67 per cent phosphorus) prepared in the cold by the method of Plósz (13) modified in minor respects by Zaleski (6), Halliburton (12), Woltering (14), Spitzer (15), and Scaffidi (10). In a later paper we shall present evidence bearing

⁷ In most cases we collected the pseudoglobulin and albumin for the purpose of subsequent chemical analysis. The former, together with the filter paper to which it adhered, was permitted to stand overnight in cold dilute salt solution. The paper was then removed and the pseudoglobulin coagulated by boiling. The coagulum was filtered off and washed with water, alcohol, and ether and dried at 105°. Likewise, the heat-coagulated albumin was washed with alcohol and ether and dried at 105°. We regularly weighed the fractions so obtained and found that the gravimetric values for albumin checked well with the values estimated from the nitrogen determinations before and after coagulation. Our gravimetric pseudoglobulin values were always much too low, but the character of the precipitate, its small quantity, and the tenacity with which it clung to the filter paper occasioned much loss.

⁸ It is so described in our preliminary paper (1).

on the relationship between pH and solubility which indicates that this protein may be properly classed among the globulins.

As for the euglobulin, pseudoglobulin, and albumin, we mention these by name in the present paper as though they were distinct chemical entities of definite and characteristic composition and properties, but this is only because of expediency and convenience. In actual fact we prefer to regard these substances as fractions and not as individual proteins. Certainly we have little reason for believing that euglobulin obtained by dialysis of the salt-soluble fraction would be identical with euglobulin obtained by salting-out. Indeed, the animal globulins, generally, show such a marked tendency to undergo dissociation (16) and reaggregation, that it is perhaps futile to regard euglobulin and pseudoglobulin as anything but products of variable composition arising from a single precursor.

It is nevertheless true that much significance of physiological import can be attached to these fractions, provided that a single method of fractionation, necessarily empirical but designed to give the very minimum of uncontrolled variables, is scrupulously adhered to from experiment to experiment.

For the purposes of quantitative analysis we have attached greater weight to the values obtained by calculation from the nitrogen determinations than to those obtained by direct weighing. Inspection of the method outlined above shows that one is obliged to resort to weighing for determination of the euglobulin and final residue but the nitrogen values alone would be sufficient for Globulin II, pseudoglobulin, and albumin. Thus the Globulin II content can in all cases be calculated from the difference between the alkali-soluble nitrogen and the Globulin II filtrate nitrogen; the pseudoglobulin values may be calculated by difference from the nitrogen determinations on the pseudoglobulin-albumin fraction and the albumin fraction; albumin may likewise be calculated from the nitrogen determinations on the albumin fraction and the albumin-free filtrate. Nevertheless the values obtained by gravimetry provided valuable checks upon the results obtained by calculation from the nitrogen determinations.⁹ It might be

⁹ Our object in resorting to bulk precipitation of Globulin II, pseudoglobulin, and albumin was not, primarily, to provide gravimetric checks upon the values calculated from the nitrogen determinations, but to pro

mentioned that the Globulin II and albumin values obtained by the two methods were in good agreement, despite the opportunities for loss attendant on resolution and reprecipitation of Globulin II. The gravimetric pseudoglobulin values were always much too low but the character of the precipitate, its small quantity, and the tenacity with which it clung to the filter paper, occasioned much loss. We had no check upon the euglobulin values, since the salt-soluble fraction prior to dialysis contained much non-protein nitrogen which was absent from the pseudoglobulin-albumin fraction.

Before presenting the results of these experiments two remaining points of considerable importance must be touched upon. One is the desirability of using blood-free livers. The quantity of blood remaining in the liver after thorough bleeding from a large artery does not seem to have been investigated but it is probably not inconsiderable and may vary greatly from animal to animal. Such blood is rich in proteins and it is evident that the quantitative significance of liver analyses would be vitiated if appreciable (and variable) quantities of blood were present. A number of the early investigators attempted to wash out the residual blood but Grund (17) appears to be the first to have fully appreciated the need of using the blood-free organ.

After the completion of many experiments (Parts A and B) it was suggested to us that the semipermeability of the liver capillaries might break down during the death of the animal, and the subsequent perfusion, however rapid and gentle, might cause a washing out of the more diffusible liver proteins (albumin). That this does not happen is demonstrated by the careful work of Grund (17), who analyzed fractions of the perfusate. Nevertheless we thought it best to run a final group of experiments upon animals which were thoroughly bled from the dorsal aorta and vena cava but in which the liver was not perfused. These results are presented in Part C and will be discussed later in this paper.

Finally we have become convinced that it is essential to carry out organ protein fractionations at a temperature as near to 0° as is feasible. The liver, above all, is very rich in proteolytic enzymes, and the globulins seem to be of unusual instability.

vide quantities of these fractions suitable for later examination with respect to nitrogen, phosphorus, sulfur, and amino acids.

Thus far we have found no effective method of inhibiting proteolysis and protein denaturation except by low temperature fractionation. Even at 0-3° proteolysis is not reduced to a negligible level. Since the rôle of proteolysis and the possibility of preventing it by the addition of inhibitors will be discussed in a later paper, we shall not mention it further at the present time.

In commencing the experiments recorded here we attempted, in the interests of simplicity, to work at room temperature. The results obtained were not without significance and are presented briefly in Part A which follows.

Results

Part A. Preliminary

Five experiments were conducted upon male rats of which one group was maintained upon the stock ration until the time of killing, two groups upon the low protein diet, and two upon the high protein diet. The fractionations were conducted at room temperature. For extraction of the salt-soluble proteins we used 5 per cent sodium chloride containing 0.2 M dipotassium hydrogen phosphate and 0.01 per cent mercuric chloride. The latter was added to inhibit proteolytic activity. The pH of the mixture, during extraction, approximated 7.6.

In contrast with subsequent results neither pseudoglobulin nor albumin was obtainable. Both were lacking or present in the merest traces. Even after 4 days dialysis the pH of the salt-soluble fraction out of which the euglobulin had settled was almost 7. Euglobulin was slightly more abundant than in subsequent experiments but settled down in the dialysis tubes in two distinct layers. This suggested gross contamination with a second fraction. On acidification to pH 4.5 to 5.0, the pseudoglobulin-albumin fraction deposited a rather voluminous precipitate, which for convenience we shall designate Fraction X.

In the next two experiments both phosphate and mercuric chloride were omitted from the salt solution used in extraction. The pseudoglobulin-albumin fractions were now almost neutral to methyl red and on a further slight acidification with acetic acid there was no precipitation of Fraction X in one case and a slight precipitation in the other. Both pseudoglobulin and albumin were now obtained in quantities fully comparable with those yielded in subsequent experiments.

We interpreted the preceding experiments as follows: Extraction at pH 7.6 or the presence of mercuric chloride reduces the solubility of the pseudoglobulin and albumin or affects unfavorably their extraction from or their formation in the liver suspension. Some Globulin II also passes into solution and comes out, in part with the euglobulin and in part as Fraction X on acidification to pH 4.5 to 5.0. Solubility experiments conducted at this point on Globulin II showed that it was of minimum solubility in a broad range between pH 3-(or less) and 5.2.

We therefore turned to sodium chloride alone as the extraction agent and adjusted the liver suspension to approximately pH 5.0 at the commencement of extraction. It seemed to us that only by extraction at a pH within the minimum solubility range of Globulin II could one obtain a salt-soluble fraction cleanly

TABLE I

Distribution of Proteins in Mammalian Liver (Gm. of Protein per 100 Gm. of Liver)

Fractionation was carried out at room temperature.

Sex	Diet	No. of animals	Globulin II	Euglobulin	Pseudo-globulin	Albumin	Final residue
Female	Low protein	18	5.09	3.31	0.59	0.71	0.34
"	High "	12	7.92	4.24	1.34	1.85	0.77
Male	Low "	13	5.59	2.98	0.94	0.77	0.52
"	High "	10	7.65	4.45	1.41	1.59	

separated from Globulin II. All later experiments have demonstrated that such is actually the case and we therefore adhere to this pH for the extraction.¹⁰ It is evident, however, that other variables which are also dependent upon the hydrogen ion concentration have to be taken into account; some of these will be discussed in a later report. Experiments were then carried out on twenty-two groups of rats: six groups of females on the high protein diet and six on the low protein diet; five groups of males on the high protein diet and five on the low protein diet. The results are summarized in Table I. Detailed findings are not presented for the reasons given in Part B.

When these experiments were far advanced, we became more and more convinced that repetition at a temperature approaching

¹⁰ Urban (18) employed 0.225 M phosphate at pH 7.

0° was desirable. Completion of the experiments showed that the total protein for which we could account (Globulin II + euglobulin + pseudoglobulin + albumin) did not exceed 15.4 gm. per 100 gm. of liver (females on high protein diet) and it seemed to us that a somewhat higher value might reasonably be expected under conditions less favorable for autolysis. We realized that the globulins and albumins, subject as they were to 4 days dialysis at

TABLE II

Fractionation of Livers at 0-3°. Perfusion Experiments

The experiments were carried out on groups of three rats in each case.

Date	Diet	Average initial weight	Average final weight	Average weight of liver	N (mg. per 100 gm. liver)					
					Salt-soluble fraction	Alkali-soluble fraction	Globulin II filtrate	Pseudoglobulin-albumin fraction	Albumin fraction	Albumin-free filtrate
		gm.	gm.	gm.						
Oct. 17	Low protein	211	185	5.4	1910	610	25	386	226	99
" 31	" "	193	176	5.0	1840	870	33	430	243	106
Nov. 14	" "	201	186	5.7	1340	1030	55	372	216	73
" 28	" "	215	186	5.6	1440	950	44	*	*	*
Dec. 13	" "	197	180	5.0	1540	780	19	382	211	66
Average.....		203	183	5.3	1610	850	35	393	224	86
Oct. 24	High protein	196	196	6.1	2300	1300	38	576	341	139
Nov. 7	" "	216	219	7.0	2150	1220	43	538	287	93
" 21	" "	192	202	7.1	2020	1200	48	628	326	101
Dec. 5	" "	213	235	8.5	2290	1170	40	628	364	97
Average.....		204	213	7.2	2190	1220	42	593	330	108

* Lost during dialysis.

room temperature, were especially apt to undergo loss by proteolysis.

If we may now interpret the experiments in the light of those reported in Part B we can see that these apprehensions were justified. The Globulin II values are substantially the same in both series but the globulin and albumin values are lower in Part A. There can be little doubt that the difference is due to autolysis.

Nevertheless, the results in Part A are qualitatively akin to those in Part B. Each of the four protein fractions shows a significant increase as we pass from livers of low protein content to those of high protein content. The percentage increases for the respective fractions are not of the same magnitude but there is no indication that any one of the proteins functions alone as a storage protein.

TABLE III

Protein Content of Livers Fractionated at 0-3°. Perfusion Experiments
The experiments were carried out on groups of three rats in each case.

Date	Diet	Gm. per 100 gm. body weight				Gm. per 100 gm. liver			
		Globulin II	Euglobulin	Pseudoglobulin	Albumin	Globulin II Calculated*	Euglobulin	Pseudoglobulin Calculated*	Albumin Calculated*
Oct. 17	Low protein	0.105	0.155	0.029	0.023	3.65	5.33	1.00	0.79
" 31	" "	0.148	0.142	0.033	0.024	5.21	5.01	1.17	0.85
Nov. 14	" "	0.187	0.083	0.030	0.027	6.10	2.71	0.98	0.90
" 28	" "	0.170	†	†	†	5.66	†	†	†
Dec. 13	" "	0.134	0.150	0.031	0.026	4.72	5.27	1.08	0.91
Average.....		0.149	0.133	0.031	0.025	5.07	4.58	1.06	0.86
Oct. 24	High protein	0.250	0.227	0.047	0.040	7.88	7.18	1.47	1.26
Nov. 7	" "	0.236	0.235	0.050	0.039	7.39	7.37	1.57	1.21
" 21	" "	0.254	0.218	0.067	0.049	7.22	6.20	1.90	1.41
Dec. 5	" "	0.256	0.239	0.060	0.061	7.06	6.59	1.65	1.67
Average.....		0.249	0.230	0.056	0.047	7.39	6.84	1.65	1.39

* Calculated from nitrogen values in Table II by use of the factor 6.25.

† Lost during dialysis.

Part B

Nine experiments were now performed with five groups on the low protein diet and four groups on the high protein diet. The extractions, precipitations of Globulin II, and dialyses were all carried out at 0-3°, according to the procedure of fractionation described on p. 498. The results are presented in Tables II and III.

Discussion of the results will be deferred until the experiments of Part C are presented.

As stated on p. 499 it was brought to our attention that perfusion of the liver might cause a washing out, not only of blood, but of the more diffusible liver proteins. We therefore undertook the experiments reported in Part C in which perfusion was omitted.

Part C

Experiments on ten groups of female rats (twenty-seven animals in all) were run under conditions identical with those prevailing

TABLE IV
Protein Content of Livers Fractionated at 0-3° Perfusion Omitted

Date	No. in group	Diet	Average weight of liver	Gm. per 100 gm. liver			
				Globulin II Calculated*	Euglobulin	Pseudo-globulin Calculated*	Albumin Calculated*
			<i>gm.</i>				
Jan. 9	2	Low protein	4.6	7.61	2.64	1.22	1.16
" 24	3	" "	4.5	8.16	3.88	1.56	
Mar. 20	3	" "	7.1	5.84	3.37		1.57
" 27	3	" "	5.9	9.27	2.93		1.61
Apr. 17	2	" "	5.0	6.00	4.42	1.31	1.60
" 29	3	" "	4.6	5.66	4.45	1.16	2.06
Average.....			5.3	7.09	3.61	1.31	1.60
Jan. 16	3	High protein	5.7	10.03	4.66	1.76	1.86
" 31	3	" "	7.6	7.08	7.59	1.78	1.89
Apr. 3	3	" "	8.4	7.54	6.66	1.63	2.50
" 24	2	" "	7.1	8.60	5.42	1.78	2.18
Average.....			7.2	8.31	6.08	1.74	2.11

* Calculated from nitrogen values by use of the factor 6.25.

in Part B except that perfusion of the liver with saline was omitted. The animals were well bled from the dorsal aorta and vena cava. The results are summarized in Table IV. For brevity we shall omit all but the most pertinent portions of the data.

DISCUSSION

As a basis for discussion the significant portions of the preceding tables are summarized in Table V.

Attention is drawn to the following points:

(a) Reduction of the fractionation temperature to 0-3° has no effect on the Globulin II values.

(b) Reduction of the fractionation temperature increases substantially the euglobulin and pseudoglobulin values, owing, in all probability, to a lessening of proteolysis during dialysis.

(c) Failure to perfuse the liver brings about an increased yield of Globulin II and albumin, probably owing to the retention of blood. The Globulin II fractions were discolored, which suggests

TABLE V
Summary (Averages Expressed in Gm. per 100 Gm. of Liver)

	Globu- lin II	Euglob- ulin	Pseudo- globu- lin	Albu- min
Room temperature fractionation				
A. Males, perfused, low protein diet.....	5.59	2.98	0.94	0.77
B. " " high " "	7.65	4.45	1.41	1.59
Ratio.....	1.37	1.49	1.50	2.06
C. Females, perfused, low protein diet.....	5.09	3.31	0.59	0.71
D. " " high " "	7.92	4.24	1.34	1.85
Ratio.....	1.55	1.28	2.54	2.60
Cold fractionation				
E. Perfused, low protein diet.....	5.07	4.58	1.06	0.86
F. " high " "	7.39	6.84	1.65	1.39
Ratio.....	1.46	1.50	1.56	1.61
G. Non-perfused, low protein diet.....	7.09	3.61	1.31	1.60
H. " high " "	8.31	6.08	1.74	2.11
Ratio.....	1.17	1.68	1.33	1.32

that hemoglobin came out in association with this fraction. The small decrease in euglobulin remains unexplained.

(d) Failure to perfuse the liver is without effect on the liver weight (Tables II and IV). Equal amounts of fluid appear to be retained, in the one case, perfusion fluid, in the other, blood. Without question the most significant experiments are those of Part B, the results of which are summarized in E and F of Table V. The equivalence of the ratios, expressive of the increases in the various fractions, demonstrates that protein enrichment within the liver, as a result of administering a high protein diet, causes equi-

proportional increases in all the proteins of the organ.¹¹ No single fraction can be regarded as a storage, reserve, or cell inclusion protein in the classical sense. The function of storage is a property of all of the hepatic proteins.

Let us mention again that we do not wish to imply that the four fractions referred to in the course of this investigation necessarily correspond with proteins resident in the liver under physiological conditions. As Halliburton (12) preferred to say, the proteins mentioned are merely "obtainable from" the liver and not, *ipso facto*, "contained therein."

Indeed the equiproportional increases in these proteins under conditions favorable for protein deposition may be interpreted in three ways:

(a) The liver contains but one or two parent proteins which dissociate (16) during fractionation with recombination of the dissociation products to give the substances actually isolated.

(b) The liver proteins constitute an equilibrium system; the formation and deposition of a single member of the system cause only its transitory accumulation; as rapidly as it accumulates it undergoes conversion into the other components of the system; a constant and characteristic proportion is thereby maintained among the members.

(c) The liver contains the four proteins actually isolated; they arise and function independently of each other.

In a later paper we shall have occasion to report experiments which bear upon these different interpretations.

It is well to note that protein enrichment within the liver is the result not only of an increased mass of tissue but of an increased content of protein per unit weight of tissue. For the 80 rats employed in all of the perfusion experiments the findings shown in Table VI were observed.

The values in the last two columns are calculated from Table III

¹¹ Throughout this paper emphasis has been given to the *increases* in protein content in passing from low protein diets to high protein diets rather than to the *decreases* in passing from the high protein diets to the low. Where the emphasis is placed is of no consequence, since the Pflüger-Voit theory not only embraces the storage of protein under conditions favorable for its deposition but also the withdrawal of protein under conditions requiring its utilization.

by summation of the four fractions. In their qualitative aspects these findings confirm the observations of Tichmeneff (4) who reported increases of 20 per cent in the liver weights of mice but of 50 to 80 per cent in the amounts of tannic acid-precipitable nitrogen in the liver.¹²

It is well to observe that the changes in the protein content of the liver under the conditions of this investigation cannot be regarded as mere reflections of corresponding changes in the protein content of other organs. The liver behaves in a manner which is qualitatively unique. In a parallel study,³ approximately 60 rats in sixteen groups were maintained upon the low and high protein diets; after exsanguination the liver, decapsulated kidneys, washed intestine, and samples of thigh muscle were weighed, immersed in boiling 0.001 N acetic acid containing about 5 per cent

TABLE VI
Results of Perfusion Experiments on 80 Rats

	Average weight of liver	Average weight of liver per 100 gm. body weight	Total protein content per 100 gm. liver	Total liver protein content per rat
	gm.	gm.	gm.	gm.
Low protein.....	5.8	3.0	11.6	0.67
High "	8.4	3.6	17.3	1.45

sodium chloride, and heat-coagulated; the samples were then ground to a paste, dried at 110°, and exhaustively extracted with alcohol and ether in a Soxhlet apparatus; the dry residues, which may be regarded as total organ protein (see also Addis *et al.* (19)) weighed as follows (percentages of initial wet weight):

	Liver	Muscle	Kidney	Intestine
Low protein.....	16.6	20.7	15.8	13.9
High protein.....	22.6	19.8	15.2	11.3

¹² Addis *et al.* (19) report that the liver protein content of young adult male rats on an adequate diet is 1.79 gm. per rat. The lower value (1.45) found in the present studies is probably due to autolytic losses during dialysis, loss during the purification of Globulin II, rejection of the final residue, and to the fact that Addis' rats were 20 per cent heavier.

Note that only the liver protein content increases on passing from the low to the high protein diet. The increase of 36 per cent compares favorably with the increase of 49 per cent (11.6 *versus* 17.3) reported in Table VI in which the total protein content was determined by direct summation of the four fractions of *soluble* protein.

Twenty-four animals on a medium protein diet gave values intermediate between those obtained from the low and high protein-fed groups.

If we assume, on the basis of the changes in body weight (*cf.* p. 493), that the weight of muscle of a rat on a high protein diet is 15 per cent greater than in a rat on a corresponding low protein diet, it follows that the total muscle protein of a rat on a high protein diet is about 10 per cent greater than in a rat on a low protein diet. The total liver protein content, on the other hand, is 120 per cent greater (last column, Table VI).

Since the kidneys are known to undergo a marked hypertrophy on high protein diets, it may be contended that these organs should also be regarded as protein depots. That this hypothesis is hardly tenable is evident from the fact that there is no protein enrichment (per unit weight of tissue) on high protein feeding. It has also been shown (19) that kidney protein in rats on adequate diets accounts for only 0.6 per cent of the total body protein, while the liver protein amounts to 4.3 per cent of the whole.

It should be mentioned that Halliburton (12) and Plósz (13) also described four or five fractions of hepatic proteins. Differentiation was based, in part, on coagulation temperatures. Blood-free livers were used and cold fractionation was attempted in some of Plósz's experiments, but we are unable to relate satisfactorily the salt-soluble proteins described in this early work to the fractions described in the present paper.

Halliburton's nuclealbumin is possibly identical with our Fraction X (*cf.* Part A). His failure to find albumin, except in the faintest traces, appears to be related to the conditions of extraction and is in harmony with the results of our introductory experiments (*cf.* Part A).

In the next paper we hope to report upon the chemistry of the liver protein fractions and shall discuss further the question of proteolysis and some of the implications that follow.

SUMMARY

1. A system of fractionation of organ proteins is described. Its application to mammalian liver gave four fractions; viz., Globulin II, euglobulin, pseudoglobulin, and albumin.

2. In rats maintained upon a low protein diet and with the fractionation conducted upon the blood-free liver at 0-3° these fractions were present, respectively, in quantities of 5.07, 4.58, 1.06, and 0.86 gm. per 100 gm. of liver.

3. In rats maintained upon a high protein diet and under the same conditions of fractionation each of the above fractions increased by 50 to 60 per cent.

4. Protein enrichment of the liver, following upon the administration of high protein diets, is associated not only with hyperplasia or hypertrophy but with an increased content of protein per unit weight of tissue.

5. The evidence suggests that none of the liver proteins can be singled out as a reserve, labile, or cell inclusion protein, in the classical sense of the Pflüger-Voit theory, to the exclusion of the basal structural proteins of the organ. All of the liver proteins participate equally in the function of storage.

The numerous nitrogen determinations required by this investigation were made by Mr. John Eudin, to whom I am also indebted for much technical aid throughout. Dr. Addis and Dr. Eloise Jameson contributed a number of valuable suggestions.

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COLORIMETRIC DETERMINATION OF ACETONE BY THE SALICYLALDEHYDE METHOD

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In some work requiring the determination of ketone bodies in blood and urine, it was decided to use the Behre and Benedict (1) method. Early in the work it was found that in comparing standard solutions of known strength, the results did not check with those theoretically expected. Moreover, comparison of tubes prepared in an apparently similar manner showed marked variations. The following work is a result of an investigation into the possible sources of error in the method.

In 1905 Frommer (2) first used the color resulting from the reaction of acetone with salicylaldehyde in the presence of strong alkali as a qualitative clinical test for acetone. Engfeldt (3) (1915) modified the qualitative test and described a rough quantitative test. Csonka (4) (1916) described a quantitative method with use of a colorimeter. The color reaction was obtained by addition of 2 cc. of a 100 per cent potassium hydroxide solution and 1 cc. of a 10 per cent alcoholic solution of salicylaldehyde to 2 cc. of the unknown and heating in a water bath at 45–50° for 20 minutes. 2 cc. of a standard solution containing 0.1 mg. of acetone in the 2 cc. were treated in the same way as the unknown. After cooling and dilution with distilled water, comparison was made with a Duboscq colorimeter.

In the Behre and Benedict (1) method, described in 1926, the color reaction is obtained by addition of 5 cc. of a 32 per cent solution of sodium hydroxide and 10 drops of salicylaldehyde to 5 cc. of the acetone-containing distillate and heating in a boiling water bath for 3 to 5 minutes. Standards containing from 0.005 mg. to 0.05 mg. of acetone are prepared at the same time. After cooling, comparison is made by colorimeter.

Korenman (5) obtains the color reaction by addition of 1 cc. of a 9 N KOH and 0.5 cc. of a 5 per cent alcoholic solution of salicylaldehyde to 1 cc. of the acetone solution and heating at 50° for 25 minutes. Comparison is made by diluting the standard until the color is equal to the unknown and then using a graph prepared by the author for calculation of the acetone content.

Several possible sources of error in the Behre and Benedict (1) method suggested themselves. (1) The presence of a definite color in the blank indicated the possibility that the final color obtained was a combination of the color due to reagents and the color due to the reaction of the reagents with acetone. (2) The salicylaldehyde is added in the form of drops—a method certainly open to objection if the amount of color obtained is closely dependent on the amount of salicylaldehyde present. (3) The solutions are heated in a boiling water bath—a temperature much above the boiling point of acetone and one at which loss of acetone due to vaporization might occur.

The presence of a color in the blank appeared to be a definite and important factor. That it was not the entire explanation, however, was shown by two findings. First, it would not explain the variations found in similarly prepared tubes. Second, the value of this factor, as determined by calculation, varied markedly and increased with higher concentrations of acetone. This is contrary to what would be expected if this factor were the entire explanation of the poor results obtained. Other possible sources of error had to be investigated first, therefore, before this one could be evaluated.

That the intensity of the final color varies markedly with the concentration of salicylaldehyde is easily determined. In view, therefore, of the necessity for accurate addition of salicylaldehyde, its addition in the form of drops is definitely open to objection. The weight of 10 drops of salicylaldehyde obtained while the dropper is held horizontally, at angles of about 30° and 60°, and vertically was determined. The results were, respectively, 0.383, 0.324, 0.275, and 0.256 gm. Three trials with the dropper at what was considered approximately the same angle (30°) gave 0.322, 0.303, and 0.347 gm., a greater variation than that due to the addition of an extra drop. The significance of these figures is self-evident.

That a significant amount of acetone might be vaporized while

the tube is being heated on the water bath is suggested by the following considerations. (1) If a tube containing 0.05 mg. of acetone, sodium hydroxide, and salicylaldehyde, as required by the Behre and Benedict method, is placed in a boiling water bath for 5 minutes and vaporized acetone tested for by the Behre (6) method, a slightly but definitely positive test is obtained. This test is negative when the tube is heated at 50° for 20 minutes. (2) Korenman found that the color developed by heating the tubes for 10 minutes at 100° was only $\frac{1}{2}$ 60 per cent of that produced by heating for the same length of time at 50°. He gives no explanation. In view of the fact, however, that development of color is more rapid at higher temperatures, the weaker color obtained at the higher temperatures suggests a possible loss of acetone, although there may be some other explanation. (3) In using the Behre and Benedict method, the reading obtained for the unknown is practically always closer to the standard than it should be, and the extent of this error is usually greater than can be accounted for on the basis of the color of the reagents alone. Such results could be explained on the basis of loss of acetone due to heating on the water bath.

It can thus be seen that at least two factors appear to be of significance in producing poor results. Before the third factor, presence of color in the blank, could be evaluated, it was necessary to modify the method to overcome the other possible sources of error. In the course of this work the following observations were made. With a given concentration of acetone the intensity of the color produced increases with increase in concentration of either sodium hydroxide or salicylaldehyde. There is, however, a certain critical concentration of salicylaldehyde for each concentration of sodium hydroxide above which a crystalline substance, probably the sodium salt of salicylaldehyde, separates out. The greater the concentration of sodium hydroxide the smaller the concentration of salicylaldehyde at which the crystalline substance settles out. For a concentration of 16 per cent sodium hydroxide, 0.2 cc. of salicylaldehyde represents approximately the largest amount which can be added to 10 cc. of solution without formation of crystals. Solutions of salicylaldehyde in sodium hydroxide deepen in color on standing. There are, therefore, two objections to the use of a combined salicylaldehyde-sodium hydroxide re-

agent. First, it does not keep well. Second, since the reagent is added to the acetone solution, the final concentration of sodium hydroxide and salicylaldehyde obtained is less than the critical concentration by the amount of dilution, and the intensity of the color obtained is less. The rapidity of development of the color increases with increase in temperature at which the tubes are heated. On cooling, the color increases, quite rapidly during the first half hour and much more slowly for a number of hours afterwards. If the tube is heated again, the color fades somewhat, to increase on cooling.

In view of the above observations it was decided to use an alcoholic solution of salicylaldehyde to insure accurate addition of the reagent and to heat the tubes at 45–50° for 20 minutes. The concentrations of the sodium hydroxide and salicylaldehyde solutions were adjusted so as to produce a final concentration in the mixture of 16 per cent sodium hydroxide and 2 per cent salicylaldehyde. When this was done, it was found that similarly prepared tubes gave similar results, and comparison of known concentrations gave results which were much closer to the calculated ones than had been the case previously. A discrepancy was still found, however, which was most evident in the comparison of the weaker solutions of acetone. If this discrepancy is assumed to be due to the presence of color due to reagents and its value calculated, it is now found to be quite constant (Table I). It might be expected that the color of the reagents would diminish as the amount of color complex increased but a glance at the relative amounts of acetone and salicylaldehyde involved makes it clear that only a very small portion of the aldehyde must be involved in the actual reaction.

On the basis of the above findings the following method for production of the color reaction and colorimetric comparison was devised. Since both Csonka and Korenman used an alcoholic solution of salicylaldehyde and heated the tubes at 50°, the method described below may be considered as a modification which embodies the following advantages. It is much more sensitive and accurate than the Csonka method in which a single standard twice the strength of the strongest standard in this method is used and in which no correction is made for the color of the reagents. It is also more sensitive than Korenman's method. The

smallest amount that can be determined by Korenman's method, 0.0025 mg. in 1 cc. (given by Korenman), is 5 or more times as large as the smallest amount determined by the method given in this paper, 0.0025 or less in 5 cc. Korenman notes the fact that the color obtained is not proportional to the acetone content and accordingly, from numerous experiments, constructs a graph showing the dilution of the standard necessary to match the color of various smaller amounts of acetone. As pointed out below, and as noted by other workers, different brands of salicylaldehyde give different intensities of color with the same amount of acetone.

TABLE I

Determination of Value of Color of Reagents (R) by Substitution in Formula 1

Acetone content of solution at 15 mm.	Acetone content of solution compared	Average reading	Value of R
mg.	mg.	mm.	
0.005	0.0075	11.52	0.00328
0.01	0.015	10.8	0.00286
0.02	0.03	10.48	0.00319

Acetone Determinations Showing Improvement in Results with Use of R

Actual acetone content	Result obtained without correcting for R	Result after correcting for R
mg.	mg.	mg.
0.0034	0.0041	0.00356
0.0071	0.00625	0.0070
0.0136	0.0122	0.0129
0.0242	0.0238	0.0244

Korenman's graphs would, therefore, be applicable only to the specific brand (not mentioned) which he used. Construction of new graphs would be necessary for each brand. Further disadvantages of Korenman's method are the marked dilution of the standard necessary for the weaker solutions of acetone and the fact that apparently side-to-side comparison is used.

Method

Solutions—

Acetone standards. A stock solution of known acetone content from which Standard A described below can be prepared is needed.

A solution containing 1 gm. of acetone in a liter of distilled water was found most useful. The simplest method for preparation of this solution was found to be as follows: A very pure grade of acetone was used (Eastman). A small flask containing some distilled water was placed on one pan of a sensitive (chainomatic) balance. 1 gm. of acetone was then added with a fine pipette, the acetone being dropped directly into the water in the flask. The acetone-containing fluid is then poured into a 1000 cc. flask, the small flask being rinsed a number of times. The volumetric flask is then filled to the 1000 cc. mark with distilled water. In our work the acetone content of this solution was always checked by the titrimetric method. The results in every case checked within 2 per cent. Once a stock solution has been prepared and its acetone content checked by the titrimetric method, it would seem possible that, provided the acetone is kept tightly stoppered and in the ice box, stock solutions might be prepared by the gravimetric method and the titrimetric check done away with. This stock solution shows no appreciable change in acetone content in 2 months.

Acetone Standard A. If the above stock solution is used, this solution is prepared by merely diluting 10 cc. of the stock solution to 100 cc. in a volumetric flask. This solution contains 0.1 mg. of acetone per cc. Behre and Benedict (1) say that this solution will last 1 month.

Acetone Standard B. Prepared by diluting 10 cc. of Standard A to 100 cc. in a volumetric flask. It contains 0.01 mg. of acetone per cc. This solution is prepared each day as needed.

Salicylaldehyde solution. Prepared by placing 20 cc. of salicylaldehyde in a 100 cc. volumetric flask and bringing it up to volume with 95 per cent ethyl alcohol. There is a marked variation (cause undetermined) in the intensity of color given by various makes of salicylaldehyde. Eimer and Amend's "Acid Salicylous, Synthetic, (Salicylic Aldehyde)" was used exclusively in this work. Other makes usually give less color. Should they be used this factor must be taken into account, as indicated below.

40 per cent sodium hydroxide solution.

Procedure

5 cc. of the solution whose acetone content is to be determined are placed in a test-tube. 4 cc. of the 40 per cent sodium hydrox-

ide solution and 1 cc. of the salicylaldehyde reagent are added. After the contents are well mixed, the tube is placed in a water bath at 45–50° for 20 minutes. It is then taken out and cooled for $\frac{1}{2}$ hour and the color compared with the standards.

Five standard tubes are prepared at the same time as the unknown. These contain 0.5, 1, 2, 3, and 5 cc. of Standard B made up in each case to 5 cc. with the necessary amount of distilled water. 4 cc. of the sodium hydroxide solution and 1 cc. of the salicylaldehyde reagent are then added and the procedure carried out as above. The standards thus contain 0.005, 0.01, 0.02, 0.03, and 0.05 mg. of acetone. If the acetone content of the unknown solution is approximately known and is within this range, 5 cc. of the unknown solution are used. If there is a possibility of the unknown solution containing more than the above amounts, it is best to make a second tube in which 0.5 cc. of the unknown solution is made up to 5 cc. with distilled water. This gives a range up to 10 mg. per cent of acetone in the unknown. Other dilutions can of course be used. Standard and unknown tubes must be made up at the same time, since, as stated above, the color continues to increase somewhat in intensity on standing.

Colorimetric Comparison—This should be done 30 minutes after the tubes have been removed from the water bath. The standard is placed at 15 mm. in a Duboscq colorimeter and should be of such strength that the unknown will give a reading between 11 and 19 mm. Readings below 15 appear to be more easily made than those above 15 mm.

Calculation—When the above procedure is used, the error due to the color of the reagents is quite constant and has a value so close to 0.003 mg. that this figure can be used in calculation of results. It must be realized that this figure holds only for the brand of salicylaldehyde used in these experiments. The color due to the reagents and the depth of color obtained on reaction with acetone will vary with the brand of salicylaldehyde used. Three different lots of the brand used in these experiments gave the same figure for the color of the reagents; it would seem safest, however, to determine the constant anew for each lot. This can, of course, easily be done by comparing solutions of known strengths and substituting in Formula 1 below.¹

¹ This is probably most easily done by comparison of tubes with 0.015 mg. against tubes with 0.01 mg. and determination of the value of R by

The amount of acetone in the 5 cc. of unknown solution is calculated as follows:

$$15 \times S + 15 \times R = a \times U + a \times R \quad (1)$$

or

$$U = \frac{15 \times S + R (15 - a)}{a} \quad (2)$$

S = acetone content of standard in mg.

a = reading of unknown

U = mg. of acetone in 5 cc. of unknown

R = factor for color of reagents (0.003 when the brand of salicylaldehyde given above is used)

When the comparison has been made with a dilution of the unknown, the result must, of course, be multiplied by the degree of dilution.

SUMMARY

A method for the colorimetric determination of acetone is described and the sources of error in a previously described method pointed out.

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substitution in Formula 1. This should then be checked by comparison of the tubes with 0.03 mg. against tubes with 0.02 mg. and again substituting in Formula 1.

THE DENATURATION AND HYDRATION OF PROTEINS. I*

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Of the different kinds of denaturation of proteins, that by heat has been investigated most thoroughly. The theory has been put forward that the loss of the zwitter ion structure by an interaction of the ionogenic groups is the most important of the electrochemical changes which a protein undergoes by the action of heat (Pauli (6), Cohn (2)). The question as to whether other structural changes are brought about by heat denaturation has been discussed by many investigators (Pauli, Haurowitz, Wu, cited in (7)) but is still unsettled at the present time. In a recent paper, Mirsky and Anson (3) presented evidence that certain groups become detectable when a protein is denatured ($-\text{SH}$ and $-\text{S}-\text{S}-$ groups), whereas they are undetectable in the natural state.

Little attention has been paid to the denaturation of proteins at an air-water interface. In a recent paper the theory has been advanced that this kind of denaturation, the surface denaturation, is closely related to the formation of surface films (Neurath (4)). Both processes involve an unfolding of the peptide chains which in the natural state are curled up in the interior of the molecule and become stretched out when the molecule comes in contact with the surface of the bulk solution. This kind of denaturation is probably important biologically because it occurs by mere shaking of a protein solution, to some extent even spontaneously.

In this paper we have investigated the differences between the denaturation by heat and by surface forces.

The affinity of a substance for the solvent is likewise an important characteristic of its structure and is conditioned by the lyo-

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philic and lyophobic groups in the molecule. A change of the structure of the protein molecule should be detectable by measuring the volume contraction in water or in solvents which have an affinity for the lyophobic part of the molecule. Groups which have the strongest affinity for water are, in the case of proteins, the ionogenic amino and carboxyl groups, the carbohydrate residues if present, the OH groups of hydroxy acids and tyrosine, and the peptide linkages. The most important lyophobic groups are the hydrocarbon residues. An interaction between the ionogenic groups in the course of heat denaturation might diminish the lyophilic properties of natural protein, whereas an unfolding of the peptide chains by surface denaturation might expose lyophobic groups to the surface, which in the natural state are buried in the interior of the molecule.

With these considerations in mind, we have measured the volume contraction of these three preparations of protein in water, alcohol, and in mixtures of both. As protein we have chosen ovalbumin from fresh hen's eggs, since the denaturation of this type of protein is irreversible, in contrast to other proteins, such as hemoglobin or serum albumin (Pauli (5), Anson and Mirsky (1)).

These experiments present strong evidence for an entirely different mechanism for these two kinds of denaturation which appear to be related to distinct changes in the state of hydration.

EXPERIMENTAL

Preparation of Material—Highly purified ovalbumin was prepared from the white of fresh hen's eggs. After the white was diluted with distilled water and the precipitated globulins were filtered off, the solution was electrodialedyzed in Pauli's apparatus until the electrical conductivity of a 5 per cent protein solution was 6×10^{-6} mho. The pH of the solution was 4.80. The solution was dried in small Petri dishes in the electric oven at 37° and then powdered in a mortar and kept in a desiccator over phosphorus pentoxide.

Heat-denatured protein was prepared by heating the purified protein solutions to 100° for 15 minutes. After the material was filtered, the precipitate was washed several times with distilled water and dried and powdered in the same way as the natural protein.

Surface-denatured ovalbumin was prepared by shaking the natural protein solution in a large flask for 48 hours in the ice room at 2°. By this procedure most of the protein was precipitated in the form of voluminous gelatinous flakes which, owing to their consistency, clogged the filter in the course of the filtration. Therefore the suspensions were centrifuged for 10 minutes, the precipitate shaken with fresh distilled water, centrifuged again, and this process repeated until no protein was detectable in the wash water. After being filtered, the precipitate showed a very gelatinous and elastic appearance. It was finally dried in the same way as the other preparations.

There was a distinct difference in the appearance of surface- and heat-denatured protein. Whereas the heat-denatured protein was a fine powder like most amorphous substances, the surface-denatured protein, powdered in the same way as the former, was fibrous and looked rather crystalline.

Both the surface- and the heat-denatured protein are insoluble in pure water, 0.1 N hydrochloric acid, 0.1 N sodium chloride, and absolute alcohol. In 0.1 N sodium hydroxide both preparations are soluble, and in about 8 per cent alcohol the surface-denatured ovalbumin shows a slight tendency to go in solution, whereas the heat-denatured ovalbumin is completely insoluble. In a subsequent paper, a thorough study of the chemical properties of surface-denatured ovalbumin will be reported.

Measurements—Density measurements were carried out with pycnometers, made of Pyrex glass, the capillary being covered with a small cap in order to prevent evaporation during weighing. Each pycnometer had a volume of about 5 cc. The densities were measured at 25° ($\pm 0.05^\circ$) and refer to water at 4°. As pycnometer liquid, xylene was found to be most suitable, having a lower surface tension and also a lower vapor pressure than toluene. Xylene was purified by drying for 3 days with phosphorus pentoxide and then distilling at 137°. The density was found to be 0.85679. Before being weighed, the pycnometers were kept in the thermostat at 25° for 20 minutes, which was sufficient for equalization of temperature. Each density was measured in duplicates which agreed within the third decimal place.

Measurements of the volume contraction of proteins were carried out by keeping the protein samples at 25° in the desiccator over

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mixtures of sulfuric acid and water of varying composition for 30 hours. One part of the moist material was used for density measurements, while in another part the water content was determined by drying the sample at 104° for 24 hours.

TABLE I
Analyses of Ovalbumin in Solid State

Substance	Density	Specific volume
Natural ovalbumin.....	1.2655	0.79020
Surface-denatured ovalbumin.....	1.3016	0.76829
Heat-denatured ovalbumin.....	1.2940	0.77280

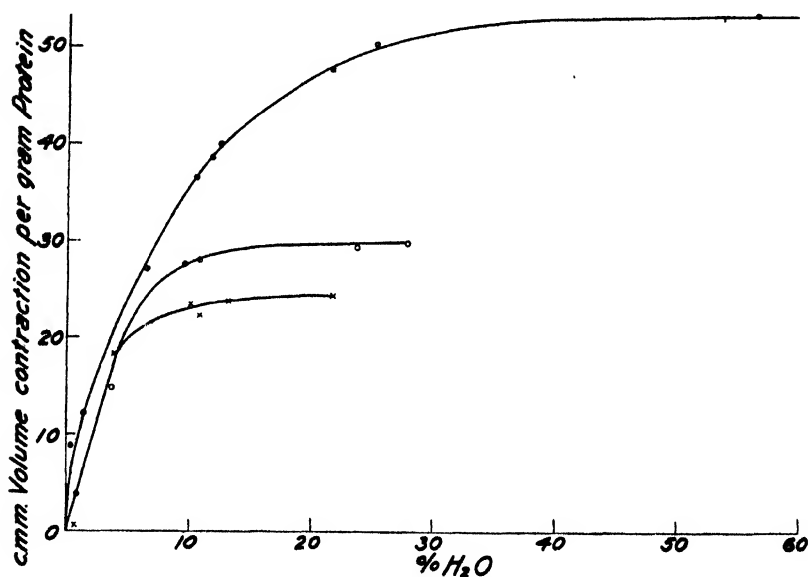


FIG. 1. Volume contraction of natural, surface-denatured, and heat-denatured ovalbumin in water. \odot natural ovalbumin, \circ surface-denatured ovalbumin, \times heat-denatured ovalbumin.

In order to measure the volume contraction in mixtures of alcohol and water, 4 cc. of the liquid in question were added to a weighed amount of protein and kept in the closed pycnometer for 24 hours. Then densities were measured in the usual way by filling the pycnometer with the same liquid which was previously

added to the dry material. Inasmuch as the density found in this manner after 24 hours was identical with that after 48 hours, we may assume that equilibrium was reached.

The concentration of alcohol in the alcohol-water mixtures was calculated from density values and refers to per cent by volume.

Results

Table I gives the values of the density and the specific volume of natural, surface-denatured, and heat-denatured ovalbumin in the solid state.

In Table II the values of the densities of the three preparations at various water contents are reported. In Fig. 1 values of the volume contraction in c.mm. per gm. of protein are plotted as ordinate and concentration of water in per cent of dry protein as

In Table III densities of surface-denatured and heat-denatured ovalbumin in absolute alcohol (density 0.78522) are given.

In Table IV are shown the densities of surface- and heat-denatured ovalbumin in water-alcohol mixtures.

In Fig. 2 values of the volume contraction in c.mm. per gm. of protein are plotted as ordinate and volume per cent of alcohol in the alcohol-water mixtures as abscissa.

DISCUSSION

From Table I we see that natural ovalbumin has a lower density than the denatured proteins. This is in keeping with Cohn's observations that with amino acids the smaller the density the greater the solubility. It is evident that if the peptide chain undergoes a stretching and uncoiling when protein is denatured at the surface, its density should be higher than that of any other possible structure, since there are no free spaces present. This is in accord with our results.

The slope of the curves in Fig. 1 allows us to estimate the total amount of water bound by the three types of protein. No further volume contraction occurs when a complete hydration of the molecule is reached or in other words when the second derivative of the volume contraction to the concentration of water approximates 0. According to our results this would give the values recorded in Table V for our preparations.

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Sørensen (8) found 0.22 gm. of water of hydration per gm. of natural ovalbumin, while Weber and Versmold (9) obtained 0.33 to 0.36 gm. For heat-denatured ovalbumin Sørensen found 0.187 gm. of water bound per gm. of protein. The slope of the curves in Fig. 1 exhibits another interesting point. Between 0 and 4 per cent water, the curves for heat- and surface-denatured protein are

TABLE II
Densities and Volume Contraction of Natural, Surface-Denatured, and Heat-Denatured Ovalbumin at Various Water Contents

Natural ovalbumin		Surface-denatured ovalbumin		Heat-denatured ovalbumin	
Per cent water	Density	Per cent water	Density	Per cent water	Density
0.0	1.2655	0.0	1.3016	0.0	1.2940
0.42	1.2784*	0.90	1.3040	0.80	1.2919
1.65	1.2791	3.74	1.3121	3.93	1.3096
6.18	1.2855	9.51	1.3111	9.98	1.2953
10.37	1.2855	10.69	1.3074	10.70	1.2916
11.70	1.2805	23.62	1.2688	13.15	1.2856
12.40	1.2809	27.88	1.2584	21.75	1.2615
21.58	1.2523				
25.09	1.2411				
56.26	1.1280				

* Determinations of densities of moist natural ovalbumin were made by Dr. Charles Rimpila.

TABLE III
Volume Contraction of Proteins in Absolute Alcohol

Protein	Volume contraction per gm. protein
	<i>c.mm.</i>
Natural ovalbumin.....	40.0
Surface-denatured ovalbumin.....	11.6
Heat-denatured ovalbumin.....	6.6

absolutely identical, whereas the curve for natural ovalbumin shows a steeper slope. Fig. 3 in which the slope of the curves (*i.e.* the volume contraction per gm. of water per gm. of protein) is plotted against the concentration of water, illustrates this relation clearly.

The curves actually represent the hydration forces and show that these forces, in the case of surface- and heat-denatured pro-

tein, are independent of the water content within 0 and 4 per cent water and show the appearance of a true hydrate.

At concentrations of water higher than 4 per cent, the curves for heat- and surface-denatured protein deviate from each other. While the heat-denatured protein gives only a small additional volume contraction, the curve for surface-denatured protein rises further, but not to such an extent as the natural protein. This might be explained if we postulate that the hydration forces of

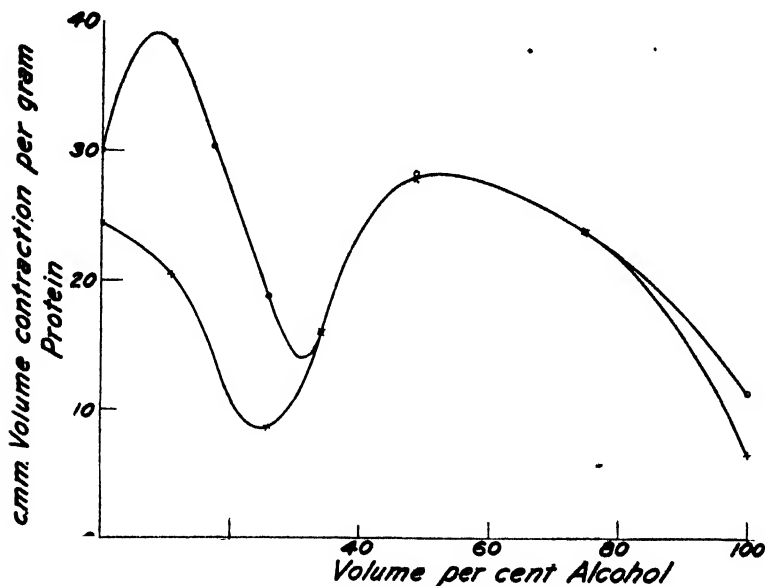


FIG. 2. Volume contraction of surface-denatured and heat-denatured ovalbumin in alcohol-water mixtures. \circ surface-denatured ovalbumin, \times heat-denatured ovalbumin.

the zwitter ionic groups are strongly diminished by the repulsion forces between the free lyophobic groups and the water molecules.

A further difference between the structure of heat- and surface-denatured protein is shown by Fig. 2 which represents the volume contraction of both samples in mixtures of water and alcohol. The maximum volume contraction of surface-denatured protein is around 8 per cent alcohol, at which concentration also the protein showed a slight solubility. Here the greatest affinity between the

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solvent and the protein is reached, probably owing to the presence of free lyophobic groups. Then, with further addition of alcohol the volume contraction decreases and after reaching a minimum it finally coincides with the curve for heat-denatured ovalbumin at 34 per cent alcohol. The volume contraction of heat-denatured ovalbumin decreases between 0 and 25 per cent alcohol, at which concentration its minimum is reached. Then, between 34 and 75 per cent alcohol both curves coincide, showing a maximum at

TABLE IV
Densities and Volume Contraction of Surface- and Heat-Denatured Ovalbumin in Water-Alcohol Mixtures

Substance	Volume per cent alcohol	Density observed	Volume per cent alcohol	Density observed
Surface-denatured.	0		33.9	1.3238
Heat-denatured.				1.3203
Surface-denatured.	10.8	1.3693	48.5	1.3520
Heat-denatured.		1.3285		1.3423
Surface-denatured.	17.3	1.3556	74.8	1.3427
Heat-denatured.				1.3352
Surface-denatured.	25.7	1.3339	100	1.3226
Heat-denatured.		1.3086		1.3050

TABLE V
Water Bound by 1 Gm. of Protein

Substance	Water per gm. protein
	gm.
Natural ovalbumin.	0.36
Surface-denatured ovalbumin.	0.19-0.20
Heat-denatured ovalbumin.	0.15-0.17

55 per cent alcohol. Finally they deviate from each other and reach the values of 11.6 and 6.6 c.mm. for surface- and heat-denatured protein respectively.

A qualitative explanation for this behavior can be given if two types of bound water are assumed as was shown by the volume contraction in water. A dehydration of the loosely bound water of heat-denatured protein occurs by the addition of small concentrations of alcohol. This would be the water bound between 4 and 20 per cent water in Fig. 1. Then, on adding further alcohol,

the volume contraction rises, owing to an additional volume contraction brought about by the binding of alcohol. With 55 per cent alcohol a dehydration of the tightly bound water takes place and the curve falls. Since this fraction of bound water is held by the same forces in both, the surface- and heat-denatured protein, between 0 and 4 per cent in water in Fig. 1, the volume contraction is identical for both samples.

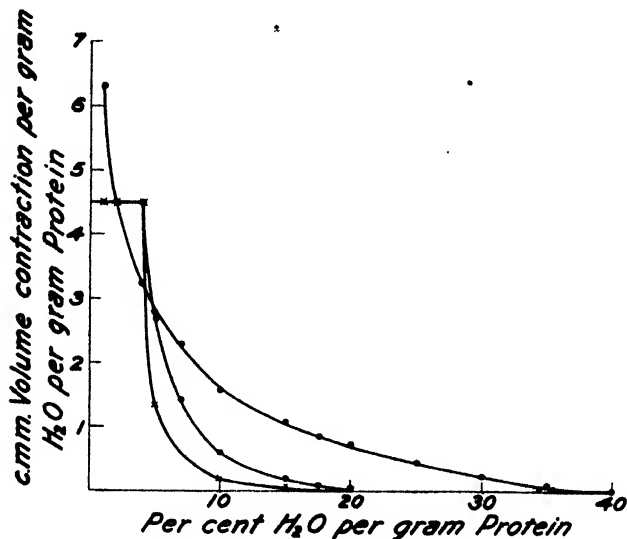


Fig. 3. Calculated volume contraction per gm. of water per gm. of protein, in water. \odot natural ovalbumin, \circ surface-denatured ovalbumin, \times heat-denatured ovalbumin.

SUMMARY

1. Densities of natural, surface-denatured, and heat-denatured ovalbumin in the dry state have been measured. Natural protein has the lowest density, while the densities of heat- and surface-denatured protein are close together, surface-denatured protein having a higher density than heat-denatured.

2. The volume contraction of these samples at various water contents has been determined. The values for the maximal volume contraction of natural, surface-denatured, and heat-denatured protein are 53, 30, and 24.5 c.mm. per gm. of material.

3. Further differences of the structure of heat- and surface-

denatured proteins are indicated by measurements of the volume contraction in mixtures of water and alcohol.

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THE VALIDITY OF DETERMINATIONS OF THE pH OF WHOLE BLOOD AT THIRTY-EGHT DEGREES WITH THE GLASS ELECTRODE

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In the past decade, considerable progress has been made in the development of various types of glass electrode apparatus for pH measurements in biological material (Kerridge, 1925; MacInnes and Dole, 1929, *a, b*, 1930; Stadie, 1929; Hill, 1931; Stadie, O'Brien and Laug, 1931; Kahler and DeEds, 1931; and MacInnes and Belcher, 1931, 1933 *a, b*). As a consequence, workers in the biological field have been quick to use the new method for finding pH values which would otherwise be unobtainable, or for obtaining results exceeding the accuracy of the colorimetric, or even of the hydrogen electrode method. For the measurement of the pH of whole blood *as drawn*, with an accuracy greater than that of the colorimetric method (Cullen, 1922, *a*; Hastings and Sendroy, 1924), the glass electrode should be especially valuable, since the hydrogen electrode is useless in the presence of oxyhemoglobin.

Although glass electrode pH measurements are appearing more frequently in the literature, no one has yet given proof of the accuracy of such values for *whole blood*, or indicated their relationship to hydrogen electrode results. Those who have attempted to give proof of the reliability of their glass electrode measurements of blood pH values (Voegtlin, DeEds, and Kahler, 1930; Yoshimura, 1935, *b*; Haugaard and Lundsteen, 1936) have demonstrated merely the reproducibility and constancy of the potentials obtained by their own particular methods of measurement. Apparently, the nearest approach to a comparative study of such material as blood is to be found in the work of Stadie,

O'Brien, and Laug (1931), who compared glass electrode with hydrogen electrode results for twenty-two specimens of *serum* at 38°, all at pH about 7.4. They used the thin membrane type (MacInnes and Dole, 1929, *a*) of glass electrode. On the average, their glass electrode results were 0.007 pH more alkaline than their hydrogen electrode values.

Haugaard (1934) has also published a parallel hydrogen electrode and glass electrode determination on one sample of serum at pH 7.4. The glass electrode result seems to be from 0.00 to 0.03 pH more alkaline than the hydrogen electrode value.

Yoshimura (1935, *b*), using Kerridge's (1925) technique, has compared pH values of five samples of whole blood, measured with hydrogen and glass electrodes. However, it does not appear that his hydrogen electrode measurements of whole blood can be accepted as valid, for the reason that they were made with blood in the oxygenated state.

Sørensen (1909) originally defined pH as the negative logarithm of the hydrogen ion concentration. More recently, there has been a tendency to regard it as a similar function of the hydrogen ion activity. MacInnes (1933) has pointed out that the first relationship is not true and that the second one cannot be proved thermodynamically. However, the usefulness of the term may be retained by defining pH simply by the equation:

$$(1) \quad \text{pH} =$$

where E in the case of the hydrogen electrode is the measured potential of the typical cell

H₂-Pt unknown liquid saturated KCl bridge reference electrode

and E_0 is a constant at any one temperature, a function of the reference electrode and a standard solution of definite, accepted pH. Equation 1 is also applied where a glass electrode is used in place of the hydrogen-platinum electrode. Even if E_0 values for both hydrogen and glass electrodes are obtained by means of the same standard solutions, up to the present time there has been no basis for predicting that both electrodes would yield the same pH values for whole blood.

Parsons (1917) showed, in *in vitro* experiments, that the pH of

reduced whole blood was the same as that of its plasma separated without change of CO_2 tension; hence for blood *as drawn* pH values in the literature have been determined in the plasma or serum. Whether colorimetrically, gasometrically, or electrometrically measured, these values have all been obtained by some reference, direct or indirect, to the hydrogen electrode. If direct blood pH values yielded by the glass electrode are to be compared with these values based on the hydrogen electrode, it appears essential that any method of glass electrode measurement used be first compared with hydrogen electrode measurements on blood. In the present paper such comparisons of pH measurements, on reduced blood, are reported.

EXPERIMENTAL

Technique

Equilibration of Blood Samples with Gas Mixtures—Ox blood, either defibrinated or with 0.2 per cent potassium oxalate plus 0.1 per cent sodium fluoride added, was used. After bleeding, the blood was kept 1 day before it was used, in order that the spontaneous lactic acid formation that occurs in freshly shed blood might come nearly or completely to an end. The blood samples were prepared for pH determinations by the double tonometer saturation technique of Austin *et al.* (1922). They were saturated, one at a time, in a water bath at 38° with gas mixtures of varying tensions of CO_2 in hydrogen, for two 30 minute periods. After separation of the gas and liquid phases, the blood sample, kept at 38° , was removed to a 38° constant temperature room where simultaneous pH determinations were made immediately by both the hydrogen and glass electrode techniques.

Hydrogen Electrode Measurements—These were carried out in a Clark-Cullen rocking electrode vessel (Cullen 1922, *b*), exactly as described for serum pH by Hastings, Sendroy, and Van Slyke (1928). For each blood sample, a different platinum electrode was used. The electrode vessel was washed and filled in each case with the same gas (by mercury displacement from the separated tonometer containing the gas phase) as that with which the blood had been saturated. Some buffer mixtures were also measured for pH in the bubbling hydrogen electrode described by MacInnes and Belcher ((1931) Fig. 2).

The E_0 value of the electrode was obtained with 0.1 *N* HCl, the

pH of which was assumed to be 1.08, or with 0.1 N acetate mixtures freshly prepared according to MacInnes and Shedlovsky (1932) and MacInnes and Belcher (1936), at pH approximately 4.5. Each acetate mixture was reproducible, on the basis of the work of these authors, to at least 0.005 pH.

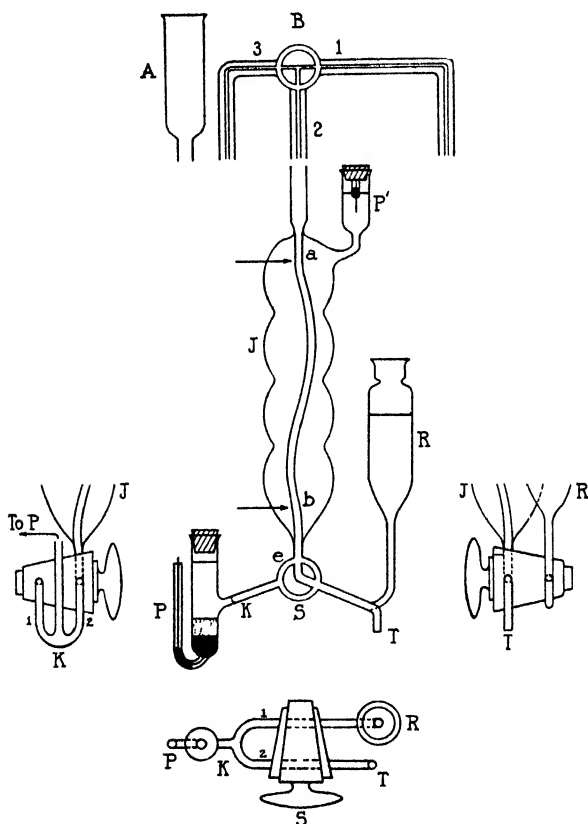


FIG. 1. Modified MacInnes and Belcher (1933, a) electrode (Electrode A).

Glass Electrode Measurements. Electrode A—Most of our measurements were made in an electrode vessel (Fig. 1) which was a modification of the rugged form described by MacInnes and Belcher ((1933, a) Fig. 1). The inner spiral was replaced by a thin tube, *ab*, slightly curved to avoid breakage with change

in temperature. For the same reason, the jacket was made of a series of bulbs. This modification of the original MacInnes and Belcher electrode increased the ease in cleaning and decreased the volume of fluid required to about 1 cc. for a tube 12 cm. long. A further improvement, for the present purposes, was effected by detaching the cup *A* so as to allow for its use interchangeably with the 3-way 1.5 mm. capillary stop-cock *B*, connecting with the vessel through transparent rubber tubing. The electrode could thus be filled from above and washed out through *T*. When buffer solutions were measured, the cup *A* was used. For CO_2 -containing solutions, or for blood or serum, the stop-cock *B* was used. The result of using this stop-cock was a saving in the amount of sample needed, the elimination of troublesome bubbles, the exposure of a minimum liquid surface to the air in the vessel, and a washing out downward of any accumulated moisture in the electrode.

* At the beginning, and sometimes also at the end of each experiment, the E_0 value of the electrode was obtained, as described by MacInnes and Belcher (1933, *a*), with the same 0.1 *N* HCl or 0.1 *N* acetate mixture used to standardize the hydrogen electrode. The E_0 values, although varying from day to day, were sufficiently constant to allow measurements to be made over a period of several hours without recalibration. In many cases, a phosphate buffer solution of known pH (according to Hastings and Sendroy (1924)), checked by hydrogen electrode measurement, was also used as a further check on the calibration of the cell.

Before the introduction of a blood sample, the electrode was rinsed several times with distilled water and allowed to drain. The rinsing water was allowed to stay in the electrode a minute or so before drainage. The moisture adherent after this last washing was sufficiently removed by the downward stream of sample subsequently introduced from *A* or *B*.

When pH was measured in blood or other CO_2 -containing solutions, the vessel containing the blood and mercury (Austin *et al.* (1922) p. 129) was attached to arm 1 of *B*. The stop-cock *B* was turned to connect arm 1 with arm 3 and a small amount of blood was allowed to waste through the stop-cock. The control stop-cock of the blood container was closed, and then with stop-cock *S* open to *T*, stop-cock *B* was turned to connect arm 1 with arm 2 and

the vessel *J*. With the stop-cock of the blood container as control, the blood sample was allowed to enter the electrode through *B*. A little blood was wasted through *S* to *T*. The blood container control stop-cock was closed, and stop-cock *S* was quickly turned clockwise through 120° to flush saturated potassium chloride from the reservoir *R* through arms 1 and 2 of stop-cock *S*, outside to *T*. By another 120° clockwise turn, a liquid junction was formed at the point *e* between the calomel electrode *P* and the sample in *ab*, through *K* and arm 2. Stop-cock *B* was then quickly turned clockwise through 180° to connect the electrode *J* with arm 3 of *B*, thus avoiding any possible strain on the glass membrane *ab*.

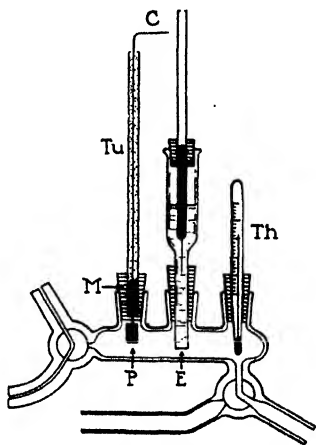


FIG. 2. Modified Clark-Cullen hydrogen electrode with MacInnes and Belcher (1933, *b*) glass electrode inserted (Electrode B).

The blood reservoir was disconnected from arm 1 of stop-cock *B* and the potential readings made.

In each experiment with Electrode A, one determination was carried out in which the tube *ab* (Fig. 1) contained room air before the blood was run in, and a duplicate determination in which tube *ab* was filled with mercury before the blood was introduced, in order to avoid any possibility of pH increase from loss of CO_2 .

Equilibrium of the blood or serum with this glass electrode, as judged by the constancy of the potential readings, was obtained, in the majority of cases, within $\frac{1}{2}$ minute, and remained constant for as long a time as measurements were taken, 10 minutes in some cases.

Glass Electrode Measurements. Electrode B—A final series of measurements was made in an electrode vessel (Fig. 2) which was simply a Clark-Cullen hydrogen electrode vessel with an additional opening for the insertion of a thin membrane, glass electrode *E* of the type used by MacInnes and Belcher ((1933, b) Fig. 1). The hydrogen-platinum electrode *P* made contact with an enameled copper wire *C* through a globule of mercury *M*. The lead-off wire *C* was held rigidly in the tube *Tu* filled with paraffin. A thermometer *Th* indicated the temperature of the solution measured. The use of this combination type of vessel made possible simultaneous hydrogen and glass electrode measurements on the same individual sample.

Results

As shown by Evans (1922), lactic acid forms in *freshly shed blood* at 38°. There is an accompanying fall in pH of about 0.03 to 0.05 during the first half hour after bleeding (Havard and Kerridge, 1929, Laug, 1930, 1934; Yoshimura, 1935, *a*; Haugaard and Lundsteen, 1936). As mentioned above, the blood used in our experiments was drawn the day before, and kept at about 10° until used for an experiment. When, in the course of the equilibration it was again warmed to 38°, the acid formation, which had presumably become slow in the cold, was still so much less than in freshly shed blood that samples kept and measured in the glass electrode at 38° for 10 minutes showed little sign of change. Drifts of ± 0.2 millivolt, likewise found for buffer solutions during this period, were no doubt caused by slight temperature changes. We were therefore satisfied that whatever acid might be formed at such a slow rate, would cause no difference in pH between the aliquots of blood used in the glass and the hydrogen electrodes, respectively, when the following procedure of preparing the blood was followed. A portion of blood (1 day old), sufficient for both electrode measurements, was warmed to 38° and equilibrated with a CO₂-hydrogen mixture of the desired CO₂ tension. Then, after separation of the gas and blood phases, separate portions of blood from the same container were transferred at once to the two electrodes. The potential measurements, completed within 5 minutes or less from the time the electrodes were filled, were made simultaneously. All operations, from saturation to pH measurements, were carried out without change of temperature at 38°.

Glass Electrode A—In the measurement of phosphate and citrate buffer mixtures, with pH values ranging from 4.0 to 7.5, no difficulty was experienced in obtaining consistent agreement between

TABLE I

Comparison of Hydrogen and Glass Electrode (Electrode A) pH Measurements on Buffer Mixtures at 38°

Material	With E_0 of electrodes obtained with 0.1 N HCl as stand- ard, pH = 1.08			With E_0 of electrodes obtained with 0.1 N acetate buffer as standard, pH = 4.510		
	Hydrogen electrode	Glass electrode		Hydro- gen electrode	Glass electrode	
	pH	pH	ΔpH	pH	pH	ΔpH
Citrate Buffer 1	b*3.67	3.67	0.00			
" " 2	4.15	4.14	-0.01			
	b 4.15					
" " 3	3.68	3.67	-0.01			
	b 3.67					
" " 4	4.15	4.15	0.00			
	b 4.15					
Phosphate Buffer 1	7.18	7.17	-0.01			
" " 2	7.36	7.36	0.00			
" " 3	7.37	7.37	0.00			
	b 7.38					
" " 4	7.48	7.47	-0.01			
	b 7.48					
" " 5	7.35	7.37	+0.02			
" " 6	7.35	7.35	0.00			
" " 7	7.37	7.36	-0.01			
" " 8	7.31	7.32	+0.01			
" " 9	7.32	7.31	-0.01			
" " 10	6.956	6.951	-0.005			
" " 11	7.154	7.159	+0.005			
" " 12	7.363	7.361	-0.002			
" " 13	7.554	7.559	+0.005			
" " 14	6.940	6.942	+0.002	6.949	6.947	-0.002
" " 15	7.139	7.151	+0.012	7.143	7.156	+0.013
" " 16	7.342	7.356	+0.014	7.351	7.361	+0.010
" " 17	7.535	7.546	+0.011	7.539	7.551	+0.012

* b = bubbling electrode.

the hydrogen electrode and the glass electrode to within 0.01 pH (Table I), with both 0.1 N HCl and 0.1 N acetate buffer as standards for the E_0 value of the electrodes.

Stadie, O'Brien, and Laug (1931) called attention to the possibility of CO₂ loss in the filling of their glass electrode. They

TABLE II

Hydrogen and Glass Electrode (Electrode A) pH Measurements on Reduced Whole Blood at 38°

Experiment No.	Sample No.	With E ₀ of electrodes obtained with 0.1 N HCl as standard, pH = 1.08			With E ₀ of electrodes obtained with 0.1 N acetate buffer as standard, pH = 4.510		
		Hydrogen electrode	Glass electrode		Hydrogen electrode	Glass electrode	
		pH	pH	ΔpH	pH	pH	ΔpH
1	1		m*7.505				
			7.500				
	2		m 7.014				
			7.021				
2	1		m 7.524			m 7.534	
		7.522	7.505	-0.017	7.526	7.515	-0.011
	2		m 6.995			m 7.005	
		7.010	6.992	-0.018	7.013	7.002	-0.011
3	1		m 7.565			m 7.562	
		7.587	7.564	-0.023	7.578	7.561	-0.017
	2		m 7.337			m 7.334	
		7.347	7.334	-0.013	7.338	7.331	-0.007
	3		m 7.186			m 7.182	
		7.204	7.181	-0.023	7.199	7.177	-0.022
	4		m 7.037			m 7.033	
		7.052	7.039	-0.013	7.047	7.035	-0.012
4	1		m 7.614			m 7.609	
		7.635	7.617	-0.018	7.610	7.612	+0.002
	2		m 7.364			m 7.369	
		7.388	7.368	-0.020	7.365	7.373	+0.008
	3		m 7.168			m 7.163	
		7.200	7.163	-0.037	7.180	7.158	-0.022
	4		m 7.005			m 7.000	
		7.027	7.001	-0.026	7.003	6.996	-0.007
5	1		m 7.167			m 7.164	
		7.203	7.162	-0.041	7.177	7.159	-0.018
	2		m 7.000			m 6.997	
		7.030	6.998	-0.032	7.004	6.995	-0.009
Average.....				-0.023			-0.011

* m = sample put into electrode by displacement of mercury.

obtained an average increase of 0.006 pH with serum samples exposed, during the filling of the electrode, to air instead of to the

equilibrium gas mixture of 40 mm. of CO_2 in hydrogen. In some experiments (marked *m* in Table II), by first filling the glass electrode (Electrode A) completely with mercury, and then introducing the sample, we obtained measurements with the possibility of CO_2 loss eliminated. The results (Table II) for the same blood samples were in agreement within narrow limits of either increased alkalinity or acidity whether the entering sample displaced mercury or air in the electrode.

The results of the comparison of blood pH measurements (Table II) indicate, with 0.1 N HCl as a standard of reference for the E_0

TABLE III

Hydrogen and Glass Electrode (Electrode B) pH Measurements on Reduced Whole Blood and Dilute Bicarbonate-Carbonate Solutions at 38°

Both electrode measurements were made simultaneously in the same vessel, with the material in equilibrium with the gas phase.

Experiment No.	Material	Hydrogen electrode	Glass electrode	
		pH	pH	ΔpH
1	Phosphate buffer	7.17	7.17	0.00
	Ox blood defibrinated	7.20	7.18	-0.02
	Phosphate buffer	7.42	7.42	0.00
	Ox blood defibrinated	7.40	7.38	-0.02
	Phosphate buffer	7.63	7.63	0.00
	Ox blood defibrinated	7.66	7.64	-0.02
2	$\text{NaHCO}_3 + \text{Na}_2\text{CO}_3$, total content from 0.007 N to 0.01 N	8.483	8.491	+0.008
		8.722	8.715	-0.007
		8.984	9.001	+0.017
		9.623	9.630	+0.007
		10.302	10.314	+0.012

value of the electrodes, an average difference of 0.023 pH in the direction of relatively more acid values for this particular glass electrode as against the shaking or rocking hydrogen electrode. With 0.1 N acetate buffer as standard, this discrepancy is reduced to 0.011 pH, still in the same direction.

Glass Electrode B—By the use of the combination glass and hydrogen electrode vessel (Fig. 2) all uncertainty as to variations in the sample itself measured by the two electrodes was eliminated. The vessel was filled with the gas mixture with which the sample had been equilibrated, and both electrode readings were taken

simultaneously after 5 minutes of gentle rocking. The results of Table III, with a fresh platinum-hydrogen electrode used for each blood sample, indicate that with 0.1 *N* HCl as a standard for the calibration of the electrodes, the blood measurements for the thin membrane type of glass electrode are also 0.02 pH more acid than those for the hydrogen electrode simultaneously immersed in the same sample. For phosphate buffer solutions measured at the same time there was complete agreement, whereas dilute bicarbonate-carbonate solutions at high pH showed an average relative deviation of +0.01 pH for the glass electrode. The results by the two types of Glass Electrodes A and B were therefore in agreement, in that blood pH values by both, with 0.1 *N* HCl as standard, were 0.02 pH more acid than by the hydrogen electrode.

DISCUSSION

In evaluating the significance of these results, it will be helpful to consider the possible sources of error involved.

Although it is not our purpose to enter into a discussion of pH standardization (Clark, 1928) we must note again that 0.1 *N* HCl, of all standard solutions used for the establishment of the E_0 for an electrode, so far as reproducibility of potentials is concerned, is less satisfactory than are the less acid buffer mixtures. Secondly, there may be some error, from 0.005 to 0.01 pH, involved in the preparation of any buffer solution.

The hydrogen electrode is extremely susceptible to the presence of oxygen or of substances giving rise to mixed oxidation-reduction potentials. This must be particularly the case when whole blood is measured. As is well known, no hydrogen electrode may be used indefinitely at 38° for reduced blood measurement. When withdrawn from use after one measurement, it is frequently coated with protein. Just when such an electrode may be considered to be in the initial stages of being "poisoned" is not known. Furthermore, although we were using blood reduced as completely as possible, in these experiments, it must be noted that removal of oxygen was not absolutely complete. Van Slyke and Sendroy (1933) found, in reduced dog blood prepared according to the equilibration technique outlined, from 0.3 to 2.0 per cent of the available hemoglobin still binding oxygen. The effect of such small amounts of oxygen in this form on the rocking hydrogen elec-

trode is not known and the possibility of error from this source must therefore be kept in mind.

The glass electrode, on the other hand, is uninfluenced by the presence of oxygen or other so called hydrogen electrode "poisons." Indeed, we have found potentials of buffer mixtures to be unaffected even when the solutions were suspended in mineral oil. However, the glass electrode has its own limitations in the exceedingly careful work required not only for constant, but for reproducible results.

When the E_0 values of glass and hydrogen electrodes were determined with 0.1 N acetate buffer, the blood pH yielded by the glass electrodes varied over the range $+0.008$ to -0.022 (mean -0.011) from the pH yielded by the hydrogen electrode (Table II). This range of deviation does not exceed that which might be expected from the combined sources of error in standardizing both electrodes and in the possible slight "poisoning" of the hydrogen electrode by blood constituents. When the electrodes were standardized with 0.1 N HCl instead of acetate, the pH values yielded by blood varied from -0.017 to -0.041 (mean -0.023) from the pH yielded by the hydrogen electrode. For determining the E_0 values of the electrodes, HCl gives less consistently reproducible results than does acetate buffer, and the lower pH of the HCl removes the conditions of E_0 standardization further from those of blood pH determination. Presumably the greater and more variable deviation between glass and hydrogen electrode results, when the electrodes were standardized with 0.1 N HCl, may be attributable to the less satisfactory nature of HCl as standard.

SUMMARY

A comparison has been made at 38° of pH values yielded by the Clark-Cullen rocking hydrogen electrode, with values yielded by two forms of glass electrode: A, the tubular form of MacInnes and Belcher (1933, *a*) and B, a MacInnes and Belcher (1933, *b*) thin membrane electrode. The latter was inserted into a Clark-Cullen electrode, so that measurements by both the glass and the hydrogen electrode were made on the same solution and at the same time. The materials used for these measurements were buffer solutions and whole blood at physiological CO_2 tensions.

• With citrate, phosphate, and bicarbonate-carbonate buffer solutions, hydrogen electrode pH values agreed consistently, within 0.01 pH, with values by both types of glass electrode.

With reduced whole blood, equilibrated at various CO₂ tensions, Glass Electrode A, gave pH values in the range +0.008 to -0.022 (mean -0.011) from the values obtained by the hydrogen electrode.

In obtaining these results the E_0 values of the electrodes, both glass and hydrogen, were determined with standard acetate buffer solutions of pH 4.5. When 0.1 N HCl was used to determine the E_0 values, Glass Electrode B consistently gave blood values 0.02 pH less than those yielded by the hydrogen electrode. Slightly less consistent results, of the same average order of magnitude, were yielded by Glass Electrode A, standardized with HCl.

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THE UTILIZATION OF GLUTATHIONE IN CONNECTION WITH A CYSTINE-DEFICIENT DIET

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Although various aspects of the metabolism of glutathione have been the subject of numerous investigations within the past few years, the intermediary steps in the catabolism of this compound remain undetermined. It is not known whether glutathione is catabolized through cleavage to the constituent amino acids and thence through the steps ordinarily involved in the metabolism of these amino acids or whether it is broken down in some unique fashion.

Experimental proof of the oxidizability of the sulfur of this tripeptide in the animal organism has been reported by Hele and Pirie (1), by Abderhalden, Buadze, and Geidel (2), by Schelling (3), and by Brand, Cahill, and Harris (4). The oxidation of the sulfur of glutathione to sulfate by tissue slices has been demonstrated by Pirie (5). No reference has been found by us, however, in relation to the availability of the cysteine of glutathione for the growth of animals on a cystine-deficient diet. Lewis and Lewis (6) have demonstrated that other peptides such as diglycylcystine and dialanylcystine may be successfully substituted for the *l*-cystine supplement to a cystine-deficient diet of rats, but the fact that glutathione differs from these peptides in containing a γ linkage suggests that its hydrolysis and consequent ability to serve for cystine in the diet should not be assumed without experimental proof. It might even be recalled that Hopkins (7), in 1921, in his original paper on glutathione called attention to the resistance of the compound to the proteolytic enzymes of the tissues. This observation led Lewis and Lewis to comment in 1927 that glutathione is probably able to function in the organism because of its

resistance to hydrolysis by enzymes and to other like metabolic changes (6).

More recent evidence based on *in vitro* enzymatic studies indicates, in fact, that the glutamic acid linkage in glutathione is not easily split by enzymes. Grassmann, Dyckerhoff, and Eibeler (8) found that glutathione in its reduced or oxidized form is not attacked by the proteases, pepsin, pancreatic protease, and papain. Glutathione proved to be resistant also to hydrolysis by dipeptidase and aminopolypeptidase from yeast and from the intestine. It was observed, however, that carboxypolypeptidase from the pancreas effected a rapid and smooth cleavage of oxidized glutathione, but that the hydrolysis came to a standstill after half of the peptide linkages in the molecule had been disrupted. They precipitated the unhydrolyzed moiety, and from the filtrate they were able to isolate glycine in more than an 80 per cent yield as the ester hydrochloride. The reduced form of the tripeptide was not significantly attacked by carboxypolypeptidase, a behavior which was attributed to the inhibiting effect of the sulfhydryl group. Kendall, Mason, and McKenzie (9) in a study of the enzymatic hydrolysis of glutathione came to the conclusion that erepsin hydrolyzes glutathione into its three constituent amino acids. In a later paper, however, Mason (10) stated, "that erepsin cannot attack the glutathione molecule at the link between the glutamic acid and cysteine." This was to be expected, he observed, since the free amino group is in the γ position. When the glutamyl radical had been split off as pyrrolidonecarboxylic acid, the resulting cysteinylglycine was easily attacked by the erepsin. In the earlier experiments, glutathione was incubated with erepsin at 37° for 2 to 5 days and apparently the glutathione was hydrolyzed by the enzyme. In the later studies Mason showed that under these same conditions, but without erepsin, pyrrolidonecarboxylic acid was split off in this manner and the cysteinylglycine was then hydrolyzed by erepsin. He therefore concluded that erepsin does not hydrolyze both peptide linkages of glutathione. It is, of course, questionable whether enough time would be afforded in the intestinal tract for this type of non-enzymatic splitting off of the pyrrolidonecarboxylic acid to take place to any significant extent. Thus, on the basis of the *in vitro* enzymatic studies, there is nothing to indicate that an appreciable amount of cysteine can be liberated from glutathione in the intestinal tract.

The work of Brand and coworkers (4) is also pertinent to the question under discussion. These investigators have compared the metabolic behavior of glutathione with that of cystine, cysteine, and methionine in the cystinuric individual, and have observed that the oral administration of glutathione or of cystine causes practically no increase in the urinary cystine, whereas the feeding of cysteine or methionine results in a marked increase in the excretion of cystine by the cystinuric. They interpreted these observations as proof of a difference in the paths of metabolism of cystine and glutathione on the one hand and methionine and cysteine on the other. They concluded moreover that this behavior of glutathione in the cystinuric argues against the hydrolysis of glutathione to furnish cysteine as an important path of its metabolism, and they suggested that reduced glutathione may be oxidized before catabolism, or that its metabolism may proceed along an entirely different and unconventional course.

We therefore felt that the hydrolysis of glutathione to its constituent amino acids either after oral or parenteral administration could not be accepted *a priori*. It was thought that a study of the availability of glutathione for growth purposes might throw some light on this question. If it were found that growth was supported, it would indicate the possibility that hydrolysis *can* take place, but of course would not indicate that glutathione *must* ordinarily follow this path in its catabolism. However, it should be pointed out that the question still obtains in these studies, as in others of this nature, as to whether the utilization of the supplement is due to its conversion in the body to the deficient factor or is only a matter of a sparing action for the small amount of cystine or methionine which is present in the diet, a question so amply discussed by Jackson and Block (11). From this standpoint a sparing action of the glutathione in this particular study could mean that the supplement might be used as such without hydrolysis for certain purposes, and that the small amount of cystine and methionine in the diet thus spared might serve for the synthesis of new protein in the tissues and for other anabolic purposes. However, a failure on the part of glutathione to support growth in lieu of cystine would conclusively demonstrate that the formation of cystine or cysteine could not take place in the catabolism of this tripeptide.

A study of the utilization of glutathione by animals on a cystine-

deficient diet was therefore undertaken. Glutathione was administered orally to some of the animals and was injected subcutaneously into others. It was found that glutathione supported growth under these conditions, apparently as efficiently as an

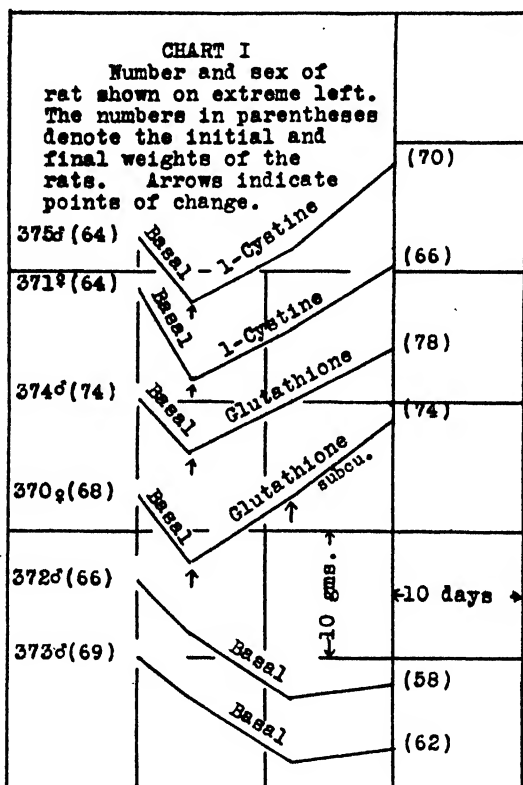


CHART I. Growth curves of rats on a cystine-deficient diet, supplemented with *l*-cystine and with glutathione.

equivalent amount of cystine. This was as true when the tripeptide was injected as when it was fed.

EXPERIMENTAL

Two litters of rats were used for the investigation. For the first litter the basal diet selected was similar to that which has

been employed previously in this laboratory. The percentage of the milk vitamin concentrate was increased and the dextrin was correspondingly decreased. A new lot of the vitamin concentrate was used and the increased amount was found necessary. The basal diet had the following percentage composition: casein 6.0, dextrin 33.0, sucrose 15.0, agar 2.0, salt mixture (Osborne and

TABLE I
Food Consumption

Rat No. and sex	Days	Daily supplement to basal diet	Daily food consumption gm.
372 ♂	1- 4		6.5
	4-20		6.3
373 ♂	1- 4		5.8
	4-20		5.3
370 ♀	1- 4		5.0
	4-20	52 mg. glutathione (oral)	8.9
374 ♂	1- 4		5.0
	4-20	52 mg. glutathione (oral)	7.1
371 ♀	1- 4		4.8
	4-20	20 mg. <i>l</i> -cystine (oral)	5.9
375 ♂	1- 4		4.8
	4-20	20 mg. <i>l</i> -cystine (oral)	7.2
506 ♀	1- 5		5.3
	5-22	1 cc. 0.9% NaCl (subcutaneous)	4.4
508 ♂	1- 5		6.8
	5-22	1 cc. 0.9% NaCl (subcutaneous)	4.7
507 ♀	1- 5		6.0
	5-22	52 mg. glutathione (subcutaneous)	6.0
510 ♂	1- 5		6.0
	5-22	52 mg. glutathione (subcutaneous)	6.4
511 ♂	1- 5		6.8
	5-22	52 mg. glutathione (subcutaneous)	7.7

Mendel (12)) 4.0, lard 19.0, cod liver oil 5.0, and milk vitamin concentrate (Supplee *et al.* (13)) 16.0. Two rats were given supplements of 20 mg. daily of *l*-cystine, two were given 52 mg. of reduced glutathione, an amount of glutathione which contained the sulfur equivalent of the cystine, and two animals were given control dextrin pills. The crystalline glutathione used in the investigation was shown by the Sullivan reaction (14) to be nega-

tive in respect to free cystine or cysteine. The supplements with one exception were given in the form of dextrin pills, and the daily dose for each animal was divided into two portions which were fed at 9 a.m. and 6 p.m. Rat 370 was given the glutathione pills as supplement for 8 days, after which the material was ad-

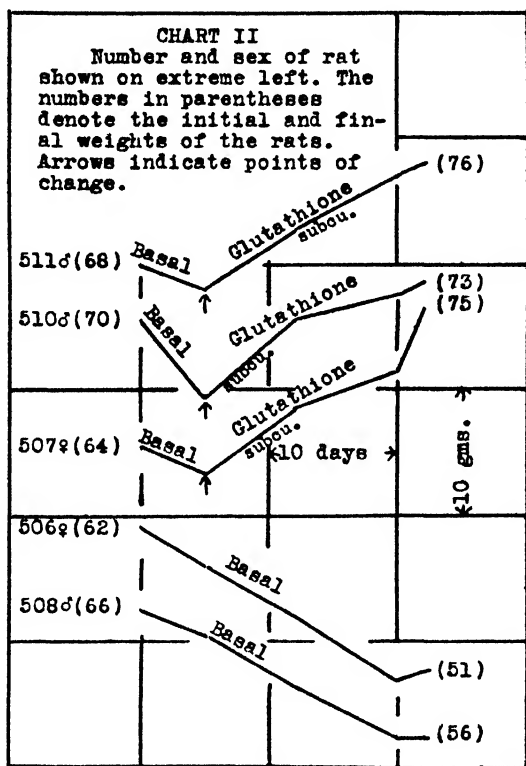


CHART II. Growth curves of rats on a cystine-deficient diet supplemented with subcutaneous injections of glutathione or of physiological saline.

ministered subcutaneously twice a day. The glutathione was dissolved just previous to injection in 0.5 cc. of physiological saline and was neutralized to litmus with NaOH. The growth curves of the animals are presented in Chart I, while the average daily food consumption for each rat is given in Table I. The rate of growth

of Rat 370 was not affected by the change from oral administration of the glutathione to subcutaneous injection. Additional experiments on the parenteral injection of glutathione were subsequently carried out with another litter of animals.

With this litter the basal diet contained 5 per cent casein and 12 per cent milk vitamin concentrate in place of 6 per cent and 16 per cent respectively. The dextrin was correspondingly increased. This afforded an even greater deficiency of cystine in the diet. Three rats were given supplements of 52 mg. daily of glutathione injected subcutaneously, as described for Rat 370 of Litter I, the daily dose for each animal being divided into two portions. Two control animals were injected subcutaneously at the same time with the same volume of physiological saline. The growth curves of these animals are presented in Chart II, while the average daily food consumption for each rat is given in Table I.

SUMMARY

The results obtained in this study demonstrate that glutathione, whether administered orally or injected subcutaneously, is able to support the growth of animals on a cystine-deficient diet, thus indicating the possibility that cystine or cysteine may be liberated from this tripeptide during metabolism.

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STUDIES IN HISTOCHEMISTRY

IX. THE QUANTITATIVE DISTRIBUTION OF VITAMIN C IN THE ADRENAL GLAND AT VARIOUS STAGES OF DEVELOPMENT

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PLATE 2

(Received for publication, June 29, 1936)

In a previous investigation (1) a correlation was made between the quantitative occurrence of vitamin C and the histological structure in the various regions of the adult bovine adrenal. From these data it was possible to estimate the quantity of vitamin C per cell in the different types of cells present. The present study is an extension of this work to include strictly analogous investigations on the bovine adrenal at various developmental stages starting with the 6 month fetal, and finishing with the calf, gland. It was found that it was not practical to study glands from those fetuses younger than 6 months because of their smaller size and very thin cortex.

EXPERIMENTAL

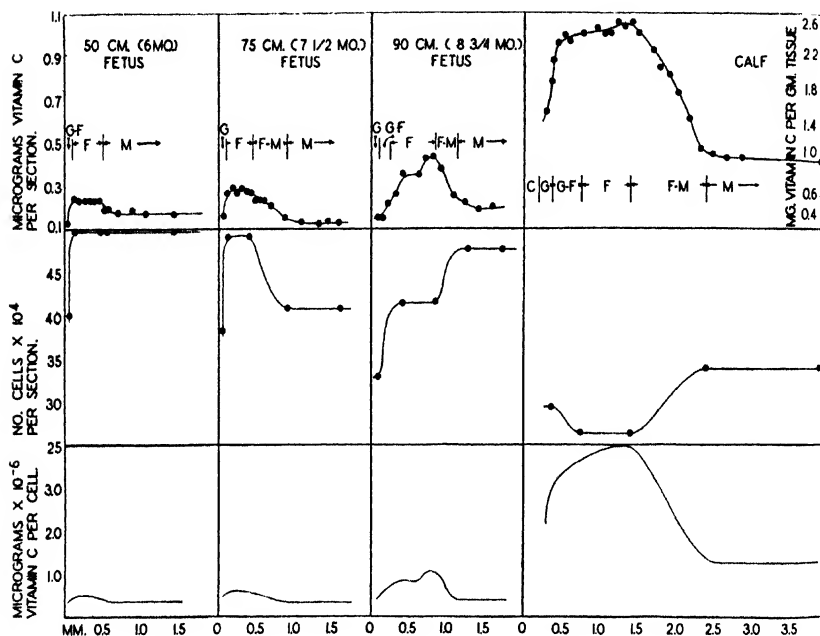
The procedures employed for the measurement of the vitamin C by titration with 2,6-dichlorophenol indophenol, the histological control work, and the cell counting technique were identical with those employed previously (1), with the exception that 2 per cent metaphosphoric acid (2) was used for the extraction of the vitamin C rather than 9 per cent acetic acid.

The results are represented by Text-fig. 1. The data, from which the points were obtained for the curves of the number of cells per section, are given in Table I. The curves presented are considered typical, since they are essentially the same as those from parallel studies on several glands in each of the age levels investigated.

DISCUSSION

Histological Changes at Various Ages

Figs. 1 to 4 show that the cortex in four periods is distinctly demarcated from the medulla and undergoes a progressive increase in size. The most active developmental changes take place in the glomerulosa. In the youngest gland it is represented by cells



TEXT-FIG. 1. Distribution of vitamin C, number of cells, and content of vitamin C per cell in the adrenal gland at various developmental stages. *G* represents glomerulosa; *F*, fasciculata; *M*, medulla; *C*, capsule; *G-F*, mixture of *G* and *F*; *F-M*, mixture of *F* and *M*.

arranged in simple arcs which straighten out to form the parallel strands of the fasciculata. This looping becomes more complex with age and finally in the calf the cells are compactly arranged in a glomerular pattern much like that of the adult. As the gland gets older the fascicular strands increase in length, and in the calf the innermost zone approaches the reticulated appearance of the adult. The medulla in these periods is composed of an outer zone

TABLE I

Cell Counts in Various Portions of Adrenal Gland

The readings represent the number of cells per 0.072 ml of stained tissue. Volume of slices titrated: 415 ml, except for the 90 cm. fetus which was 444 ml.

Source of tissue	Cell counts	Average count	Cells per slice
50 cm. fetus			
Glomerulosa	95, 97, 126, 102, 116, 112	108	$\frac{108 \times 415}{0.112} = 400 \times 10^3$
Fasciculata	129, 135, 145, 129, 141, 137	136	$\frac{136 \times 415}{0.112} = 504 \times 10^3$
Medulla	121, 138, 148, 137, 129, 145	136	$\frac{136 \times 415}{0.112} = 504 \times 10^3$
Shrinkage factor 1.16. $0.072 (1.16)^3 = 0.112$ ml equivalent in fresh tissue			
75 cm. fetus			
Glomerulosa	127, 105, 107, 108, 119, 126	115	$\frac{115 \times 415}{0.125} = 382 \times 10^3$
Fasciculata	151, 142, 159, 142, 135, 162	148	$\frac{148 \times 415}{0.125} = 491 \times 10^3$
Medulla	113, 111, 125, 115, 138, 136	123	$\frac{123 \times 415}{0.125} = 408 \times 10^3$
Shrinkage factor 1.20. $0.072 (1.20)^3 = 0.125$ ml equivalent in fresh tissue			
90 cm. fetus			
Glomerulosa	98, 85, 97, 96, 89, 96	93	$\frac{93 \times 444}{0.125} = 330 \times 10^3$
Fasciculata	106, 123, 117, 122, 124, 110	117	$\frac{117 \times 444}{0.125} = 415 \times 10^3$
Medulla	135, 132, 128, 142, 145, 139	135	$\frac{135 \times 444}{0.125} = 479 \times 10^3$
Shrinkage factor 1.20. $0.072 (1.20)^3 = 0.125$ ml equivalent in fresh tissue			
Calf			
Glomerulosa	92, 98, 108, 116, 95, 92	100	$\frac{100 \times 415}{0.141} = 294 \times 10^3$
Fasciculata	99, 91, 93, 86, 81, 92	90	$\frac{90 \times 415}{0.141} = 264 \times 10^3$
Medulla	114, 119, 111, 112, 116, 121	115	$\frac{115 \times 415}{0.141} = 338 \times 10^3$
Shrinkage factor 1.25. $0.072 (1.25)^3 = 0.141$ ml equivalent in fresh tissue			

of compactly arranged cells showing an intense chromaffin reaction, and an inner zone in which there are scattered cells that later differentiate into adult cells of the sympathetic system. This differentiation has taken place in the calf. Numerous sympathetic nerves and dilated veins and sinusoids are present in the inner portion of the medulla at all stages.

Vitamin C Changes at Various Ages

Examination of the curves of the concentration of vitamin C in the various regions of the adrenal gland (Text-fig. 1) shows in all cases maximum values in the fascicular region of the cortex. Since there is no reticular zone in the glands at these age levels, and an absence of a less dense region in the fascicular zone, these curves have a shape different from that given for the gland of an adult animal (1), which has both the reticular zone and the less dense fascicular region.

Variations in the relative size of the cells in the different portions of any gland account for the lack of a consistent shape for the curves of the number of cells per section. However, the general trend of these curves is to fall to a lower level with increasing age of the gland because of the enlargement of the cells with development. The shape variations in these curves are not great enough to disturb the character of the other curves representing the quantity of vitamin C per cell, all of which have the same general shape. These latter bottom curves represent the ratio of the upper to the middle curves, and it may be seen that with increasing age there is a rise in the amount of vitamin C per cell. This is the result of two additive factors which enter as development proceeds—the increasing size of the cells, and the increasing concentration of the vitamin.

Examination of comparable data in the adult animal (1) shows an extension of these tendencies. The number of cells per section was lower, hence the size of the cells greater, and the quantity of vitamin C per cell was greater than in the calf gland. This latter effect existed in spite of the fact that the concentration of vitamin C was lower in the adult than in the calf gland.

The differences in the distribution of vitamin C in the cortex may be explained by the fact that connective tissue-like cells in the capsule differentiate to form glomerular cells which continue their

growth inward to form the fasciculata and finally die in the reticularis (3). The fascicular cells which have the greatest store of the vitamin probably have the greatest functional activity. Apparently the actively growing glomerulosa cells store less of the vitamin.

SUMMARY

The quantitative distribution of vitamin C, and the number of cells per unit volume throughout the various regions of the bovine adrenal gland have been determined at developmental stages from the 6 month fetus to the calf. From this the quantity of vitamin C per cell was estimated for the different types of cells present.

The fascicular region of the adrenal at all stages of development contained the highest concentration of the vitamin. The medulla and glomerular zone contained concentrations of the same order of magnitude. The concentration increased in all regions with the development of the gland, reaching a maximum in the calf and falling off in the adult animal. Both the size of the cells and the quantity of vitamin per cell increased regularly from the fetal to the adult stage of the animal.

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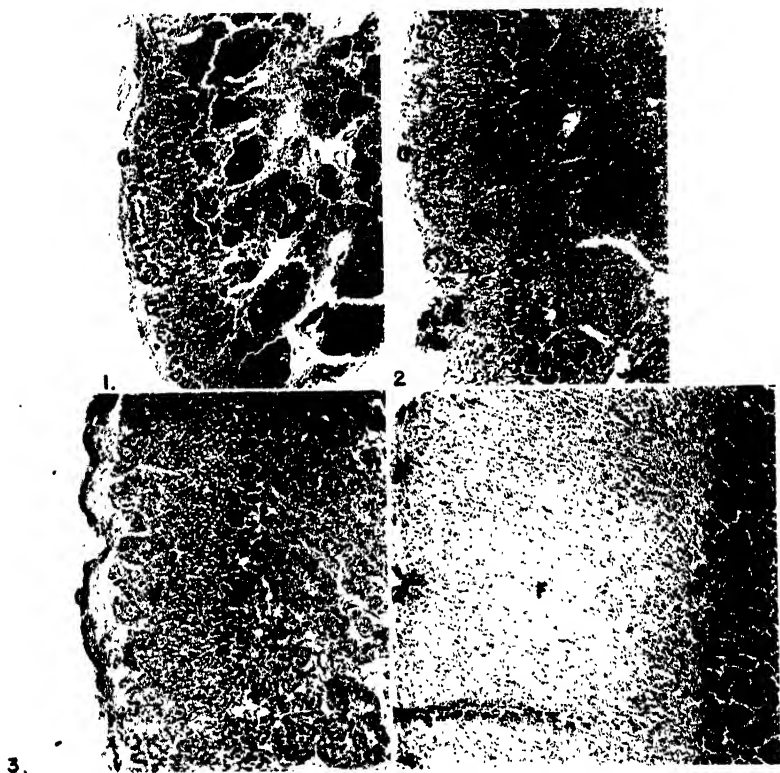
EXPLANATION OF PLATE 2

FIG. 1. Representative cross-section of the adrenal gland of a 50 cm. fetus; $\times 25.5$. *G* represents glomerulosa; *F*, fasciculata; *M*, medulla.

FIG. 2. Representative cross-section of the adrenal gland of a 75 cm. fetus; $\times 25.5$. The meaning of the letters is the same as for Fig. 1.

FIG. 3. Representative cross-section of the adrenal gland of a 90 cm. fetus; $\times 25.5$. The meaning of the letters is the same as for Fig. 1.

FIG. 4. Representative cross-section of the adrenal gland of a calf; $\times 25.5$. The meaning of the letters is the same as for Fig. 1.



(Glick and Biskind: Vitamin C in adrenal gland)

THE MULTIPLE NATURE OF THE THIRD FACTOR OF THE VITAMIN B COMPLEX

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(Received for publication, June 6, 1936)

The availability in crystalline form of both vitamin B and vitamin G (flavin) has enabled the deficiencies resulting from a lack of these factors to be fairly well characterized. Much confusion, however, exists with regard to the "third factor," or residuum necessary in addition to vitamins B and G (flavin) to complete the vitamin B complex. Investigations have been made of the relation of the third factor to the prevention or cure of pellagra (1), black tongue (2-4), rat dermatitis (2, 5, 6), and the dermatitis caused by feeding chicks a heated diet of natural foodstuffs (7, 8) and referred to in this paper as "chick dermatitis." In many of these investigations fullers' earth has been used as an adsorbent for fractionation. Some of the results obtained with fullers' earth adsorbates and corresponding filtrates are shown in Table I. Sometimes the material adsorbed by fullers' earth has been fed as an eluate.

Table I shows that adsorbates and filtrates have both been reported effective for the prevention of canine black tongue and rat dermatitis. Only filtrates have been found to cure chick dermatitis. The probability of a single factor being involved is therefore less in the case of canine black tongue or of rat dermatitis than in the case of chick dermatitis.

The present communication shows that the dual nature of the third factor may be demonstrated by fractionation with fullers' earth. It has been found that vitamins B and G (flavin) may be readily removed from a solution such as an aqueous extract of rice bran by means of a relatively small amount of fullers' earth.

Further treatments with fullers' earth remove, much less readily, a third factor (13), related to the prevention of rat dermatitis. There remains in solution another factor, the "filtrate factor" (14), which prevents chick dermatitis. For convenience, the factor preventing rat dermatitis will be referred to as Factor 1 and the filtrate factor, preventing chick dermatitis, as Factor 2. With the recognition of two factors other than vitamin B and vitamin G (flavin), it also seemed of importance to examine the source of vitamin B (85 per cent alcoholic extract of white corn)

TABLE I

Activity of Fullers' Earth Adsorbates (or Their Eluates) and Corresponding Filtrates in Prevention or Cure of Dermatitis Due to Deficiency of "Third Member" of Vitamin B Complex

Syndrome tested	Source of aqueous extract	Activity of fullers' earth adsorbate	Activity of filtrate
Canine black tongue	Yeast	+ (9)	
	Rice polishings	+ (10)	
	Liver	- (3)	+ (3)
Rat dermatitis	Yeast	+ (11)	+ (12)
	Wheat germ	+ (13)	
Chick dermatitis	Rice bran	-*	+ (8)
	Liver	- (7, 8)	+ (7, 8)
	Whey	- (14)	+ (15)

The figures in parentheses represent bibliographic citations.

* Present investigation.

used by Goldberger and Lillie (11), to characterize, if possible, the nature of the deficiency encountered by these workers.

EXPERIMENTAL

Methods

The basal diet for rats consisted of sucrose 59 parts, casein 27, lard 10, Salt Mixture 185 (16) 4, and 2 drops daily of cod liver oil.¹ After 10 days on the basal diet, daily supplements were fed of 6 units of a vitamin B concentrate² and 40 micrograms of

¹ The cod liver oil was furnished by Mead Johnson and Company, Evansville, Indiana, by courtesy of Mr. Masterman.

² Furnished by Dr. Elmer H. Stuart of The Lilly Research Laboratories, Indianapolis. We are especially grateful for this gift, without which the work would have been impossible.

crystalline vitamin G (lactoflavin).³ The supplements were apparently free from the third factors. The diet⁴ and technique used with chicks have been described elsewhere (8, 14).

Factor 1 was prepared as follows: 100 cc. of concentrated aqueous rice bran extract (8),⁵ found by assay to contain about 50 international units of vitamin B per cc., were diluted with 3 volumes of water and shaken with 50 gm. of fullers' earth to remove vitamins B and G. The first adsorbate was rejected, and the filtrate was treated again with fullers' earth to give a second adsorbate. The second adsorbate was eluted with 750 cc. of 0.2 N barium hydroxide solution. Sulfuric acid was added to bring the eluate to a pH of about 8.5, and the solution was then concentrated under reduced pressure to a thin syrup, which was precipitated several times with methanol to remove inert matter. The resultant solution was freed from barium by the addition of sulfuric acid, and concentrated under reduced pressure.

Factor 2 was prepared by diluting 100 cc. of liver extract (17) with 3 volumes of water, and shaking with 40 gm. of fullers' earth.⁵ This treatment with fullers' earth was repeated five times. The final solution was concentrated under reduced pressure to the original volume. Similar concentrates were prepared from rice bran extracts.

An alcoholic extract of white corn was prepared by shaking 170 kilos of corn for 1 hour with 150 liters of 85 per cent alcohol and filtering in a filter press. The clear filtrate was concentrated under reduced pressure to about 12 liters, dried on 4.25 kilos of corn-starch, and ground. Lumps of zein were present, which were discarded because they could not be readily ground. As a source of vitamin B, 10 per cent of this material was incorporated in the basal rat diet to replace an equal amount of sucrose.

Results

Fig. 1 shows the growth obtained when Factors 1 and 2 were fed separately and together to rats already receiving vitamins B and

³ We are indebted to the Vitab Products, Inc., of San Francisco, for the vitamin G concentrate from which crystalline vitamin G (lactoflavin) was prepared, and for the rice bran extract.

⁴ Casein was generously supplied by Mr. J. Chrisman of the California Milk Products Company, Gustine, California.

⁵ Some of the liver preparations were made from liver extract (17) kindly prepared for us by Dr. F. Fenger of Armour and Company, Chicago.

G (flavin). Addition of Factor 2 alone resulted in slow growth, and the rats developed an acute dermatitis (Fig. 2). The paws, ears, area around the mouth, and, at times, the eyelids, were affected. If Factor 1 was added at this stage, growth was quickly restored, and the dermatitis was cured. The response to Factor 1

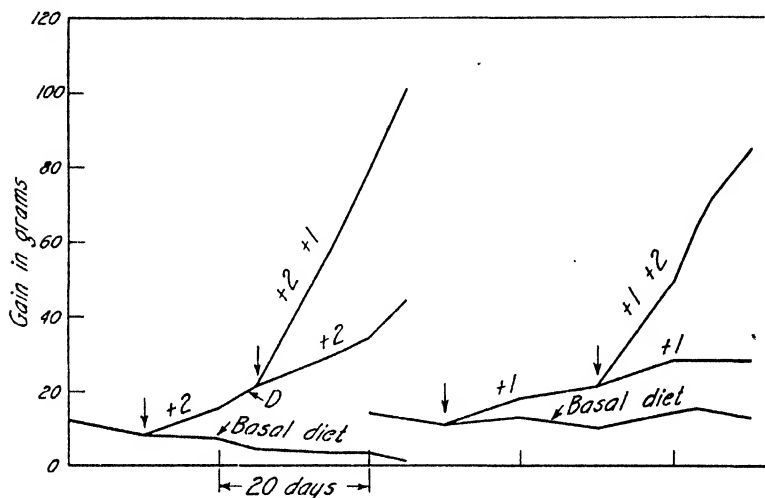


FIG. 1. Effect of Factors 1 and 2 on the growth of rats fed on a basal diet supplemented with vitamins B and G (lactoflavin). 1 indicates daily addition of 0.1 cc. of Concentrate K-48, supplying Factor 1; 2 indicates daily addition of 0.1 cc. of Concentrate K-50, supplying Factor 2; D indicates the appearance of dermatitis. Rats on the basal diet did not develop dermatitis. On the basal diet plus Concentrate K-50 an acute dermatitis developed (Fig. 2). This was cured by Concentrate K-48. On the basal diet plus Concentrate K-48, the eyelids became swollen and were stuck shut. The eyes were watery and the nose was inflamed. Concentrate K-50 cured these symptoms. Each of the six growth curves represents two rats, with the exception of the basal diet curve, which represents three rats and five rats respectively. Only two of the five rats represented by the second basal diet curve survived to the end of the experiment.

was prompt and striking. Addition of Factor 1 alone did not permit growth, and some of the rats developed swollen eyelids which tended to stick together. The eyes were watery and the nose was inflamed. Factor 2 restored growth and prevented or cured this eye condition when fed at this point. The response to Factor 2 was not rapid. Without the addition

of either Factor 1 or Factor 2, most rats died without symptoms. A few developed mild dermatitis.



FIG. 2. Dermatitis due to lack of Factor 1. The rat shows dermatitis of the paws, ears, and area around the mouth and eyes. It received a diet containing adequate amounts of vitamins B and G (flavin), and a concentrate supplying Factor 2.

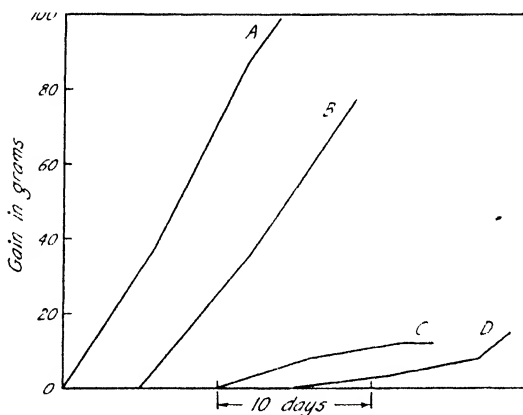


FIG. 3. Effect of Concentrate K-50, supplying Factor 2, and Concentrate K-48, supplying Factor 1, upon the growth of chicks fed a heated diet (7, 14). Ten chicks were used in each group. Curve A, basal diet plus 10 per cent of rice bran filtrate (8) (positive control diet); Curve B, basal diet plus 1.0 per cent of Concentrate K-50; Curve C, basal diet plus 1.3 per cent of Concentrate K-48; Curve D, basal diet. The syndrome score (8) at the end of the 14 day test period was for the group represented by Curve A, 0; Curve B, 0; Curve C, 16; Curve D, 8.

It may be seen from Fig. 3 that Factor 1, which cured rat dermatitis, had no curative effect on chicks when added to the heated

diet, but seemed to intensify the dermatitis slightly. Factor 2 readily cured chick dermatitis and restored growth when added to the heated diet (7, 14). Thus chick dermatitis was cured by Factor 2, which seemed actually to intensify rat dermatitis.

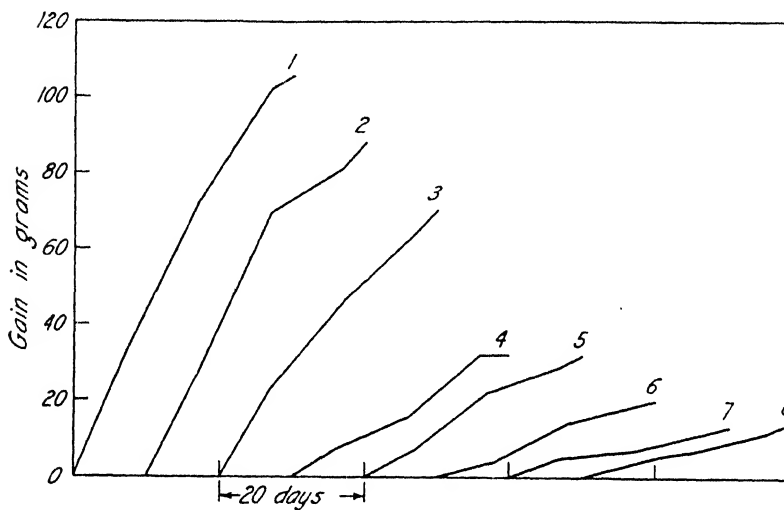


FIG. 4. Growth of rats on a basal diet containing Goldberger's alcoholic extract of white corn as a source of vitamin B, and supplemented daily with fractions supplying other members of the vitamin B complex as follows:

Curve 1, 20 micrograms of crystalline vitamin G (lactoflavin) and 0.05 cc. of Concentrate K-6, supplying Factors 1 and 2; Curve 2, 20 micrograms of crystalline vitamin G (lactoflavin) and 0.10 cc. of Concentrate K-50, supplying Factor 2; Curve 3, 20 micrograms of crystalline vitamin G (lactoflavin) and 0.10 cc. of Concentrate K-48, supplying Factor 1; Curve 4, 40 micrograms of crystalline vitamin G (lactoflavin); Curve 5, 0.10 cc. of Concentrate K-50, supplying Factor 2; Curve 6, 0.10 cc. of Concentrate K-48, supplying Factor 1; Curve 7, 0.05 cc. of Concentrate K-6, supplying Factors 1 and 2; Curve 8, basal diet. Curves 1 to 7 are composite growth curves of two rats in each group; Curve 8, of four rats.

Fig. 4 shows the results obtained with the vitamin B extract from white corn, prepared by the method of Goldberger and Lillie (11). On the basal diet, containing the extract alone, rats grew very little, and developed a dermatitis similar to that described for a deficiency of vitamin G (flavin) (18). Addition of extracts

containing Factors 1 or 2, either separately or together, did not produce growth significantly greater than that obtained on the basal diet. Addition of vitamin G (lactoflavin) alone was similarly ineffective. However, when vitamin G was added in addition to Factor 2, or to Factors 1 and 2, excellent growth was obtained. Vitamin G *plus* Factor 1 gave fairly good growth. It appeared that the deficiency in the diet was primarily that of vitamin G (flavin). It is difficult to explain by these results the rat dermatitis which was observed by Goldberger and Lillie. The acute symptoms described by these workers did not appear in the present investigation, but the rats developed a dermatitis similar to that caused by a deficiency of vitamin G (flavin) (18). It is possible that the corn used in these experiments was richer in Factor 1 than the corn used by Goldberger and Lillie. It may also be that the experiment was not continued long enough for the development of acute symptoms.

DISCUSSION

It is difficult at present to correlate the recent reports on the vitamin B complex (2, 3, 5, 6, 19). It is therefore thought advisable to limit the discussion principally to the observations made in the present investigation.

The separation of the third factor into two components makes it apparent that there are at least four components in the vitamin B complex. The search for other possible members of the complex is facilitated by this characterization.

The apparently uncomplicated nature of chick dermatitis, caused by a heated diet (7), was of great help in the present work. At the beginning of the investigation extracts containing Factor 2 free from Factor 1 could not be shown to have any biological potency with rats, but biological potency could be readily shown with chicks. Once such extracts were obtained, the basis was laid for obtaining Factor 1, free from Factor 2, by means of rat tests. Preparations containing Factor 1 could then be assayed with rats whose diets were supplemented with extracts containing Factor 2 free from Factor 1. Such diets produced acute dermatitis, cured by preparations containing Factor 1. Verification of the fact that preparations of Factor 1 were free from Factor 2 could be made by showing that these preparations, although

potent in the cure of rat dermatitis, had no effect on chicks when fed in conjunction with the heated diet (7, 14).

It is appropriate to mention some additional experimental details in fractionation: (a) Liver extract is relatively easily freed from Factor 1 by shaking with fullers' earth. (b) Rice bran extracts are not always freed from Factor 1 by this treatment. (c) Crude fullers' earth eluates prepared from rice bran often are heavily contaminated with Factor 2, as shown by chick and rat tests. This may be due to difficulty in washing adhering liquors from the fullers' earth, or to slight adsorption of Factor 2 and subsequent concentration. (d) Charcoal gives such discordant results that its use has been abandoned for the present. (e) Coprophagy has sometimes interfered with rat experiments. Dermatitis would sometimes clear up spontaneously in some rats. The skin condition, however, never became completely normal, nor was there any appreciable restoration of growth.

The experiments with a vitamin B concentrate similar to that used by Goldberger and Lillie indicated that these workers encountered a multiple deficiency. The symptoms which they described (11) confirm this indication. In the present investigations, the good growth obtained with rats (Fig. 4) on the Goldberger vitamin preparation, when supplemented with vitamin G (flavin) *plus* Factor 2 alone, or even with vitamin G *plus* Factor 1 alone, contrasts with the poor growth obtained with rats on the Eli Lilly vitamin B preparation, when similarly supplemented (Fig. 1). The results indicated that the Goldberger vitamin B supplement furnished traces of the third factors, particularly Factor 1. It is evident, however, from Fig. 4, that the third factors did not improve the Goldberger diet unless vitamin G (flavin) was added. Apparently, therefore, the primary deficiency encountered by Goldberger and Lillie was that of vitamin G (flavin).

The factor referred to in this communication as Factor 1 presumably corresponds to György's "vitamin B₆," since György and coworkers have defined "vitamin B₆" as "that part of the vitamin B complex which is responsible for the cure of the specific dermatitis developed by young rats fed on a vitamin B-free diet supplemented with purified vitamin B₁ and lactoflavin"⁶ (13).

⁶ Their definition does not provide for the multiple nature of the "third factor" and for the two syndromes brought about in rats by deficiencies of its respective components.

Factor 2 is "vitamin B₂" of Elvehjem and Koehn (7) (the filtrate factor (14)).

SUMMARY

1. An investigation was made of the "third factor," which is necessary, in addition to vitamins B and G (flavin), to supply the requirements of the rat for the vitamin B complex.

2. The third factor was found to consist of two components. The first of these was adsorbed on fullers' earth to separate it from the second. Details of the separation procedure are described.

3. The first component, Factor 1, prevented or cured an acute dermatitis in rats, but had no effect on the dermatitis produced in chicks by feeding a heated diet of natural foodstuffs (7).

4. The second component, Factor 2, prevented or cured the chick dermatitis, but when fed in absence of Factor 1, aggravated the rat dermatitis. Both components were needed by the rat for growth. Symptoms in rats of a deficiency of Factor 2 are described.

5. A study of the deficiency described by Goldberger and Lillie was made by feeding rats an alcoholic extract of white corn as a source of vitamin B. The results indicated that the deficiency encountered was multiple in nature, but that vitamin G (flavin) was the primary factor lacking.

Our thanks are due to K. K. Miya for assisting with the care of the chicks.

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STUDIES OF PHOSPHORUS OF BLOOD

V. A COMPARATIVE STUDY OF ACID AND ENZYMATIC HYDROLYSIS OF THE ACID-SOLUBLE ORGANIC PHOSPHORUS, WITH PARTICULAR REFERENCE TO THE PHOSPHOGLYCERATE FRACTION

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About 60 per cent of the phosphorus of blood consists of organic compounds soluble in dilute acid, the so called acid-soluble organic or ester phosphorus. This fraction of blood phosphorus has been further separated into at least three components, two of which, a pyrophosphate (1-8) and a phosphoglycerate (9, 10), have been isolated from blood. The remainder is thought to be composed of one or more hexosephosphates (1, 11-13).

The above compounds have been studied also by means of their relative rates of hydrolysis, either with acid or with enzymes. It has been shown that the pyrophosphate is completely hydrolyzed by boiling acid within 7 to 15 minutes (2, 14-17), that the hexosephosphate fraction is less readily hydrolyzed (16, 17), and that the phosphoglycerate is very resistant to hydrolysis (9, 10). Unfortunately, conditions chosen by the various investigators have varied so widely that considerable uncertainty still exists, particularly in regard to the relative rates of hydrolysis of the hexosephosphate and phosphoglycerate fractions. Investigations of the enzyme hydrolysis agree only in that all the phosphoric esters can be split to some extent by phosphatase (10, 18-23). It seemed advisable, therefore, to study the complete hydrolysis of the ester phosphorus fraction of blood under controlled and readily reproducible conditions. The hydrolysis of phosphoglycerate isolated from pig blood and of the commercial products β -glycerophosphate and hexosediphosphate (candiolin) was studied

under the same conditions as was blood. The commercial preparations mentioned serve as readily available controls.

EXPERIMENTAL

Normal adults were used to furnish the blood filtrate for the investigation. Into 80 cc. of ice-cold 4 per cent trichloroacetic acid in a 100 cc. volumetric flask, blood was allowed to run directly from the vein to the volume mark. Thus no anticoagulant was necessary and this concentration of trichloroacetic acid, while adequate for precipitation of the protein at the temperature used, was sufficiently low to avoid the inhibitory effect of the trichloroacetate ions on enzyme hydrolysis (23). Phosphoglyceric acid, as the barium salt, was obtained from pig blood according to the method of Greenwald (9) and converted into the sodium salt which is readily soluble in water. The hexosediphosphate¹ and β -glycerophosphate were commercial preparations.

The solutions of phosphoglycerate, hexosediphosphate, and β -glycerophosphate were made to simulate the blood filtrate as closely as possible. Because sodium sulfate was a contaminant of the phosphoglycerate and affects the rate of enzyme hydrolysis, it was added in equal concentration to the other solutions. 100 cc. of each solution contained 4 to 8 mg. of organic phosphorus, 0.5 to 1.0 mg. of inorganic phosphorus added as sodium hydrogen phosphate, 4 mg. of sodium sulfate, and 3.2 per cent of trichloroacetic acid. Aliquots of these solutions, the blood filtrate, phosphoglycerate, β -glycerophosphate, and hexosediphosphate were used in all subsequent experiments, both acid and enzyme hydrolysis.

For acid hydrolysis, hard glass test-tubes, 250 \times 20 mm., graduated at 10 and 20 cc., were fitted with small glass condensers. The condensers, ground to fit the tubes, served as stoppers, preventing evaporation and eliminating the possibility of increased pressure which occurs when tubes are sealed and immersed in boiling water as advocated by some investigators. 2 cc. aliquots of the solutions under investigation were pipetted into test-tubes, 0.5 cc. of 10 N sulfuric acid added to each, and the walls of each tube washed down with exactly 2.5 cc. of distilled

¹ Provided through the courtesy of the Winthrop Chemical Company, Inc.

water. The condensers were fitted and the tubes were placed in a water bath heated to 100° by means of an electric plate. The bath and electric plate were large enough to keep the temperature constant. At definite time intervals, tubes were removed, cooled in iced water, and the phosphorus determined (24). The total phosphorus in the solution, the inorganic phosphorus at zero time and at the chosen time interval were determined throughout all the experiments according to the method given in a previous publication (25). From the total organic phosphorus and the inorganic phosphorus formed from it during the course of acid hydrolysis, the per cent of hydrolysis at a given time was determined.

For enzyme hydrolysis, phosphatase was prepared from lamb kidney according to the method recommended by Kay (26), the enzyme being extracted from kidney by grinding with sand and water saturated with chloroform. The activity of the phosphatase thus prepared was standardized (26), 1 unit being the amount of enzyme which at 38° in Sørensen's buffer at pH 8.9 liberates 1 mg. of phosphorus from excess β -glycerophosphate in 2 hours. The extract of phosphatase was diluted to contain 150 units per 100 cc. and the same concentration of enzyme was used throughout all the experiments. The activity was checked during each experiment since phosphatase is not stable.

Enzymatic hydrolysis was carried out at pH 8.8, which has been reported optimal for kidney phosphatase (19, 26, 27), and also at pH 7.4 to approximate the pH of the blood. The pH of the substrate to be used, blood filtrate, phosphoglycerate, β -glycerophosphate, or hexosediphosphate was adjusted first with solid NaOH, then an equal volume of sodium diethylbarbiturate buffer solution (28) of the desired pH was added, and the resulting solution well mixed. In each of the series of tubes were placed 10 cc. of the substrate buffer solution, 1 cc. of phosphatase extract, and 1 drop of chloroform. Two tubes of solution were used as controls, and the remainder were stoppered and placed in an incubator, kept at exactly 38°. The contents of the control tubes were treated immediately with 4 cc. of 10 per cent trichloroacetic acid and filtered. Total phosphorus and inorganic phosphorus were determined in duplicate on samples from each tube, correction being made for the phosphorus present as an impurity in the phosphatase extract. At each desired interval of time, two

tubes were removed from the incubator, the phosphatase in the solution precipitated by the addition of trichloroacetic acid, and the inorganic and total phosphorus determined on aliquots of the filtrate. From the total organic phosphorus and the inorganic phosphorus formed by the action of the enzyme on the organic phosphorus, the per cent of hydrolysis was determined. Experiments were conducted in this way for all solutions at both pH 8.8 and pH 7.4.

In these investigations with phosphatase, effort was made to carry out the experiments under comparable conditions rather than under optimum conditions; hence, from the data one cannot state the hydrogen ion or salt concentration most favorable for the phosphatase activity on a particular substrate. The concentration of trichloroacetate ion during the time the enzyme was active was 1.4 per cent, a concentration below that found to inhibit the activity of the enzyme (23). Magnesium has been reported to alter the activity of phosphatase (27, 29, 30) but the magnesium content of the solutions studied was not determined, inasmuch as it would probably be approximately the same in all of the solutions. These solutions contained a relatively high concentration of sodium sulfate. This salt has a retarding effect on phosphatase, as was determined by comparing the relative rates of hydrolysis of solutions containing varying amounts of sodium sulfate.

In agreement with most investigators, we did not find phosphocreatine in blood. No difference was observed in the inorganic phosphate content of blood whether the blood was allowed to flow into iced trichloroacetic acid and the determination carried out immediately, or whether trichloroacetic acid was added at room temperature and the resulting mixture allowed to stand for 15 to 20 minutes before filtering. In a trichloroacetic acid solution, pyrophosphate apparently does not disintegrate into the inorganic ortho form during this time. The total phosphorus and inorganic phosphorus values of both the warm and cold solutions were the same; therefore phosphocreatine was considered absent from the blood.

The results of the hydrolysis of blood filtrate, phosphoglycerate, hexosediphosphate, and β -glycerophosphate with N sulfuric acid at 100° are shown in Chart I. Boiling blood filtrate with acid

induced a very rapid breakdown of the organic phosphorus compounds during the first 10 minutes. The 11.4 per cent split within this time probably represents, principally, the cleavage of pyrophosphate. After this initial rapid hydrolysis, the rate decreased progressively until the 12th hour. Then hydrolysis proceeded at a slow but constant velocity, indicating that all the organic phosphorus compounds in the blood filtrate are destroyed

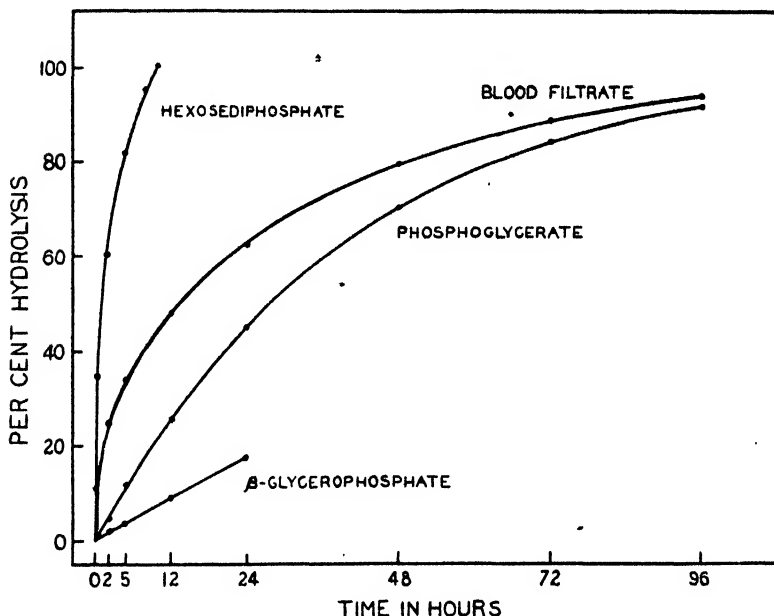


CHART I. The hydrolysis of the phosphoric esters of blood filtrate, of blood phosphoglycerate, of β -glycerophosphate, and of hexosediphosphate with $N H_2SO_4$ at 100° .

by the 12th hour with the exception of one which is fairly resistant to acid. According to the literature, this compound is said to be a phosphoglycerate.

The rate of hydrolysis of the phosphoglycerate isolated from pig blood was identical with that of the blood filtrate after the latter had been hydrolyzed for 12 hours. After this time, the organic phosphorus remaining in the blood filtrate and the phosphoglycerate undissociated after 12 hours of acid treatment are

broken down in the same manner by further hydrolysis. This is shown in Chart II. The similarity of the two compounds can be most clearly demonstrated by analyzing, from the standpoint of chemical kinetics, the hydrolysis rates of the phosphoglycerate isolated from pig blood and the organic phosphorus compound remaining in the filtrate from human blood after 12 hours hydrolysis. The time required to complete a certain fraction of a reaction is dependent upon the order of that reaction. If the

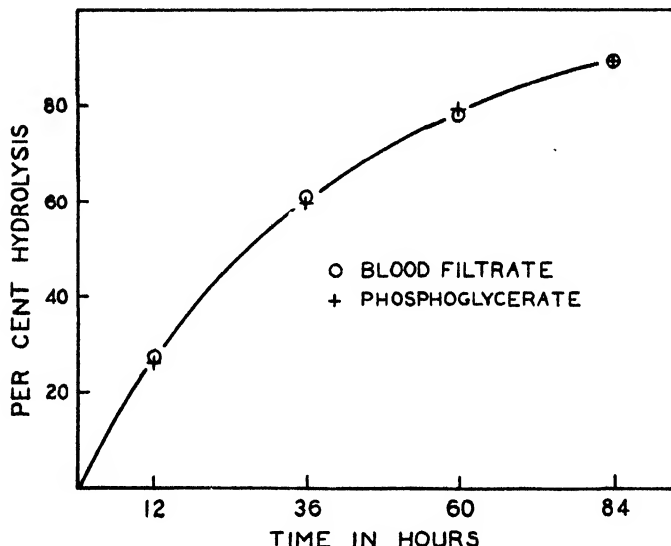


CHART II. The hydrolysis of the organic phosphorus of blood filtrate and of the blood phosphoglycerate with $N H_2SO_4$ at 100° after 12 hours preliminary acid hydrolysis.

order of the reaction is monomolecular, the value of K , the velocity constant, is independent of the initial concentration and the formula $dx/dt = K(A-x)$ can be used. By this formula, the average K for the phosphoglycerate isolated from pig blood is 0.0251. The formula does not hold for blood filtrate until after the 12th hour (Table I). From then on, K is 0.0253, showing that the compound left in the filtrate from human blood after 12 hours hydrolysis is identical with the phosphoglycerate actually isolated from pig blood in that both compounds apparently

hydrolyze in a monomolecular manner. The order of these reactions may be shown graphically also. Plotting the log of the concentration ($A - x$) against time should give a straight line if a reaction is of the first order. A straight line is obtained by plotting the data from the acid hydrolysis of the phosphoglycerate isolated from blood. The data for blood filtrate after the 12th

TABLE I

Velocity Constants of Acid Hydrolysis of Phosphoric Esters of Blood Filtrate and of Blood Phosphoglycerate As Determined by the Formula,
 $dx/dt = K(A - x)$

Material hydrolyzed	Time	x, per cent hydrolyzed	(A - x) (100 - per cent hydrolyzed)	K	Log (A - x)
	<i>hrs.</i>				
Blood filtrate	0	0	100		2.000
	0.17	11.4	88.6	0.7200	1.947
	2	24.8	75.2	0.0882	1.876
	5	33.9	66.1	0.0428	1.820
	12	48.1	51.9	0.0344	1.715
	24	62.3	37.7	0.0264	1.576
	48	79.7	20.3	0.0250	1.308
	72	89.0	11.0	0.0247	1.041
	96	94.1	5.9	0.0251	0.771
Phosphoglycerate	0	0	100		2.000
	0.5	1.2	98.8	0.0242	1.995
	2	4.8	95.2	0.0255	1.979
	5	11.9	88.1	0.0258	1.945
	12	25.6	74.4	0.0241	1.872
	24	45.1	54.9	0.0252	1.740
	48	70.4	29.6	0.0249	1.471
	72	84.2	15.8	0.0254	1.199
	96	91.6	8.4	0.0254	0.924

hour of hydrolysis not only give a straight line but the line parallels that given by phosphoglycerate (Chart III).

The above results demonstrate that both the isolated compound and the acid-resistant compound of human blood seem to be identical and that their hydrolyses proceed in the same manner as would the hydrolysis of a monophospho ester. Greenwald (9) concluded from chemical analysis that the phosphoglycerate isolated from pig blood contained 23 per cent of phosphorus and

was a diphospho ester. Chemical analysis of the substance used in the studies reported herein was unsatisfactory because of the difficulty in drying it. If the phosphoglycerate is a diphospho ester, as the chemical evidence of Greenwald indicates, the monomolecular character of the hydrolysis suggests that the two phosphoric acid groups are split off at approximately the same rate.

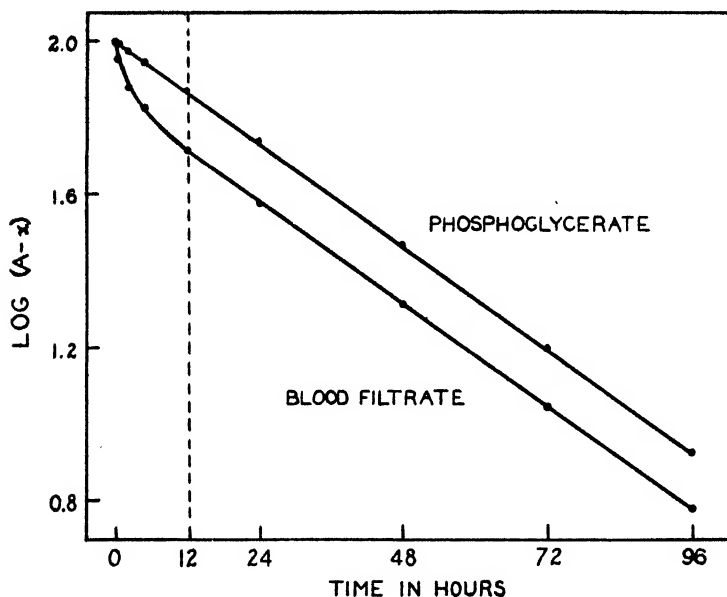


CHART III. The order of the reaction of the acid hydrolysis of the phosphoric esters of blood filtrate and of blood phosphoglycerate as determined by plotting the log of the concentration ($A - x$) against time.

The amount of phosphoglycerate normally present in human blood may be determined from the hydrolysis rates of the blood filtrate and of the phosphoglycerate from the blood. The hydrolysis rates of the two solutions have shown that a single organic phosphorus compound is left in blood filtrate after 12 hours acid hydrolysis, that this compound is split at a constant rate equal to the rate of cleavage of phosphoglycerate, and that the compound will react to acid from the beginning of hydrolysis in a way identical with the phosphoglycerate. Thus, from the amount of

phosphorus left in the blood filtrate at any interval of time during the course of hydrolysis, after the 12th hour, and the amount of phosphoglycerate remaining unchanged at the same interval of time, the per cent of blood phosphorus difficultly split with acid can be determined. For example, if the ratio of undissociated organic phosphorus at 48 hours to the undissociated organic phosphorus at zero time is the same in each solution, then the ratio for pure phosphoglycerate is 29.6:100 (Table I) and the ratio for blood filtrate must be 20.3:X or 20.3:68.5. Bloods from forty-five normal human adults were studied and the per cent of hydrolysis determined at various intervals of time after 12 hours. An average value of 68 per cent phosphoglycerate phosphorus was observed, with a range of 62 to 75 per cent.

Jost (10) has reported 75 to 80 per cent of phosphoglycerate in human blood, Kay and Robison (18) 64 to 86 per cent, Bomskov (17) 56 per cent, and Greenwald (9) 22 per cent. The method of obtaining these values has been different in each case. Greenwald isolated the material as the barium salt and Jost isolated the brucine salt. Incomplete isolation or incomplete separation of the various insoluble organic phosphorus compounds would introduce error. Bomskov, using acid hydrolysis, assumed that all the organic phosphorus remaining after 3 hours was probably a phosphoglycerate and that none of it was attacked during the 3 hour period. Kay and Robison considered the fraction resistant to enzyme hydrolysis at pH 8.8 to be the phosphoglycerate, but Roche (19) has shown that no phosphorus compound in the blood is absolutely impervious to some action by phosphatase.

Assuming that about 68 per cent of the organic phosphorus in blood filtrate is in the form of phosphoglycerate and that the pyrophosphate is destroyed within 10 minutes, blood from human adults containing an average of 21.2 mg. of ester phosphorus per 100 cc. (25) could be fractionated into 68 per cent or 14.4 mg. of phosphoglycerate phosphorus and 11.4 per cent or 2.4 mg. of pyrophosphate phosphorus (Table I). The remainder, 10.6 per cent or 4.4 mg., would be the fraction at present designated as hexosephosphate phosphorus. Mai (13) and Lawaczek (31) found about 1 per cent hexosephosphate in blood. Such a method of fractionating the organic acid-soluble phosphorus compound of blood is subject to some error and the method has been tried only

with blood of normal adults, but it may aid in determining gross changes in the ester fraction of blood in diseases wherein the phosphorus metabolism is altered.

The rate of hydrolysis of the β -glycerophosphate was much slower than that of the blood phosphoglycerate; the commercial hexosediphosphate was completely hydrolyzed within 9 hours, the "hexosephosphate" fraction of the blood, within 12 hours.

Enzyme Hydrolysis

The hydrolysis rates of the compounds possibly present in blood filtrate were determined also by means of phosphatase. (Charts IV and V). The phosphoglycerate isolated from blood was hydrolyzed with much greater facility at pH 7.4 than at pH 8.8. Asakawa (32) has reported that the optimum pH for the hydrolysis of the phosphoglycerate in blood is 7.7. The organic phosphorus compounds of the blood filtrate also were hydrolyzed more rapidly at pH 7.4 but the amount of inorganic phosphorus liberated in the blood samples as a group varied widely, especially after 24 hours. Despite the range of values, it is significant that the esters of each individual blood were hydrolyzed more rapidly at pH 8.8 during the first 24 hours, then more rapidly at 7.4. This suggests that part of the organic phosphorus compounds of blood filtrate are more easily broken down at pH 8.8 and that after these particular compounds are almost wholly destroyed, a compound is left which, like phosphoglycerate, is more readily hydrolyzed at pH 7.4. The results of enzyme hydrolysis thus support the acid hydrolysis findings. Roche (19) observed that phosphatase from blood will hydrolyze blood esters and phosphoglyceric acid at the same optimum pH. The results obtained in the present investigation are compatible with those of Kay (26) who conducted experiments for 3 hours and found that the blood filtrate esters were split by phosphatase more easily at pH 8.8. The results are also compatible with the conclusion of King (20) that two groups of esters exist in blood, one of which is more easily hydrolyzed near neutrality and the other, in an alkaline medium.

The experiments with phosphatase hydrolysis of β -glycerophosphate and hexosediphosphate confirm the findings of others (13, 26, 27, 33). Both commercial compounds are split so much

more rapidly at either pH than are the phosphorus compounds in the blood filtrate at any time during the course of enzyme hydrolysis that the existence of large amounts of these readily hydrolyzable substances in blood is improbable. While investigations with enzymes have contributed to an understanding of the nature of the phosphoric esters in blood, no adequate method has been established whereby these esters can be separated.

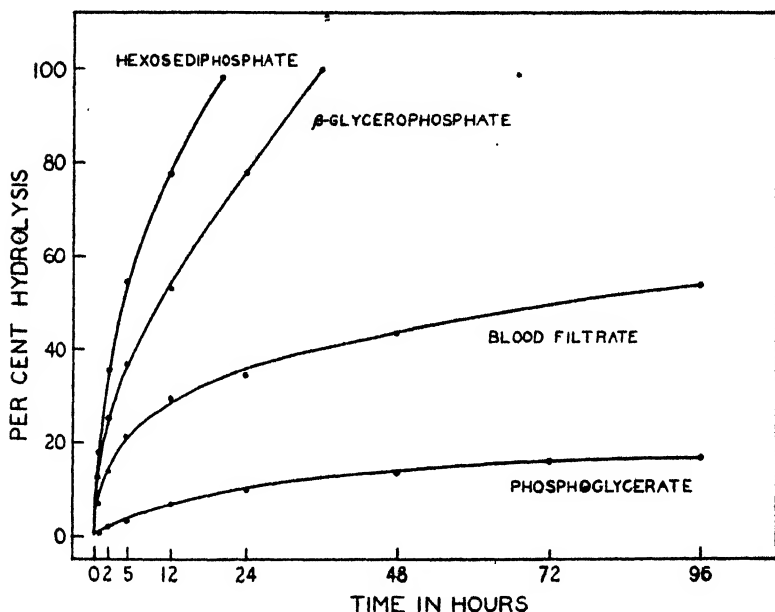


CHART IV. The hydrolysis of the phosphoric esters of blood filtrate, of blood phosphoglycerate, of β -glycerophosphate, and of hexosediphosphate with kidney phosphatase at 38° and at pH 8.8.

From the previous discussion of acid hydrolysis, it has been demonstrated that prolonged heating with acid destroys all but one organic phosphorus compound in the blood filtrate and that this residual compound may be identical with the phosphoglycerate isolated from pig blood. Blood filtrate hydrolyzed with acid for 24 hours might be expected to respond to enzyme treatment as does the isolated phosphoglycerate.

To test the validity of such a premise, blood filtrate esters were

hydrolyzed with acid 24 hours, one aliquot of the solution adjusted to pH 8.8, another to pH 7.4, and subjected to phosphatase action as in the original enzyme experiments. At pH 7.4 the hydrolysis was more extensive than at pH 8.8 but at either pH the hydrolysis proceeded 10 to 20 per cent faster after preliminary acid treatment. This was true despite the high amount of inhibitory sodium sulfate which resulted from adjusting the pH.

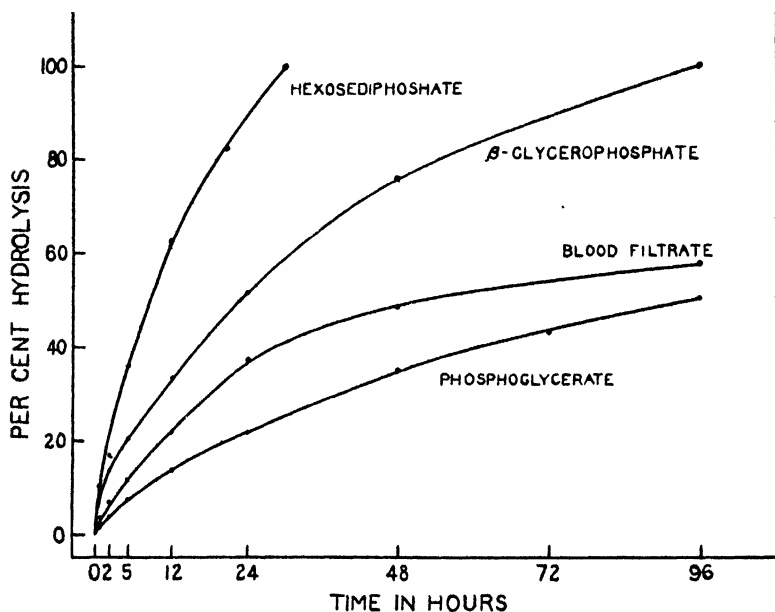


CHART V. The hydrolysis of the phosphoric esters of blood filtrate, of blood phosphoglycerate, of β -glycerophosphate, and of hexosediphosphate with kidney phosphatase at 38° and at pH 7.4.

Phosphoglycerate was treated with acid for 24 hours and then with enzyme at pH 8.8 and 7.4. Similarly, hydrolysis proceeded faster at pH 7.4 than at pH 8.8 and more readily at either pH after preliminary acid treatment. The explanation for the increased rates of enzyme hydrolysis after preliminary hydrolysis with acid is not known. The acid may alter the compounds so that the enzyme can attack them more readily, or if the phosphoglycerate is a diphospho ester, the enzyme and acid may act

selectively on the two phosphoric acid linkages. The conclusions to be drawn from an experiment of such nature are limited but the fact that both the isolated phosphoglycerate and the phosphoric ester in blood filtrate after heating with acid responded to enzyme activity more readily at pH 7.4 than at pH 8.8 supports the belief that they are identical compounds.

SUMMARY

Blood filtrate containing the acid-soluble phosphoric esters, phosphoglycerate isolated from blood, hexosediphosphate, and β -glycerophosphate were subjected to acid and enzyme hydrolysis under the same conditions of temperature, salt content, trichloroacetate ion and hydrogen ion concentration.

The hexosediphosphate was destroyed very rapidly and the β -glycerophosphate very slowly by acid hydrolysis.

The acid hydrolysis of the phosphoric esters of the blood filtrate proceeded very rapidly for 10 minutes, progressively slower until the 12th hour, then at a slow but constant rate.

The velocity constant for the decomposition of the organic phosphorus in blood after 12 hours acid hydrolysis was the same as for phosphoglycerate isolated from blood, suggesting that the acid-resistant compound in the filtrate from human blood is identical with the phosphoglycerate isolated from pig blood. The reaction is shown to be monomolecular; therefore the phosphoglycerate may be a monophospho ester or a diphospho ester in which both the phosphoric acid groups are split off at approximately the same rate.

Further evidence of the identity of the isolated compound with that present in blood was shown by comparing the phosphatase hydrolysis of each. The phosphoglycerate isolated from pig blood was hydrolyzed by the enzyme with greater ease at pH 7.4 than at pH 8.8. The organic phosphorus of blood filtrate also was hydrolyzed more readily at pH 7.4 after the 24th hour. Before that time, the blood phosphoric esters were split more easily at pH 8.8.

Hydrolysis of hexosediphosphate and β -glycerophosphate occurred readily at either pH 7.4 or 8.8 but more rapidly in the more alkaline medium.

The per cent of organic phosphorus in the blood that exists

as a phosphoglycerate can be determined by comparing the relative rates of acid hydrolysis of the organic phosphorus of the blood filtrate and the phosphoglycerate isolated from blood. Pyrophosphate has been shown to be hydrolyzed by acid within the first 10 minutes.

By calculation, the acid-soluble or ester phosphorus fraction of the blood of normal adults consists of about 68 per cent phosphoglycerate phosphorus, with a range of 62 to 75 per cent, and approximately 11.4 per cent pyrophosphate phosphorus. The remainder may be a hexosephosphate.

This method of fractionation is offered as an aid in the determination of gross changes in the phosphoric esters of whole blood during health and disease.

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THE RÔLE OF DIVALENT METALS IN THE REVERSIBLE INACTIVATION OF JACK BEAN HEMAGGLUTININ

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Concanavalin A, one of the four crystallizable globulins of the jack bean, has been identified by us as a hemagglutinin (1, 2). This hemagglutinin, the first to be isolated, promises to reveal much of interest regarding certain features of protein chemistry. It agglutinates the erythrocytes of the horse, rabbit, dog, cat, rat, and guinea pig in high dilution; it agglutinates also corn and rice starch granules and some emulsified fats. It precipitates glycogen and certain mucoproteins.

We have noted that the ability of concanavalin A to agglutinate erythrocytes and starch granules and to precipitate glycogen is lost after treatment with acid followed by neutralizing, but on standing returns completely, provided the acid has not been too concentrated or the temperature too high (3). Owing to the ability of concanavalin A to precipitate glycogen in flocks, it is rather easy to determine the time necessary for complete inactivation by acid at a given temperature and the time required for reactivation. Fig. 1 shows the minutes necessary for concanavalin A to become reactivated sufficiently to flocculate glycogen after standing at various temperatures with hydrochloric acid for different periods of time. Here we added 2 cc. of 0.118 N hydrochloric acid to 1 cc. (3.2 mg.) of concanavalin A dissolved in saturated sodium chloride. For reactivation we added 3 cc. of a solution containing 2 cc. of 0.1 N sodium hydroxide and 1 cc. of 9.6 per cent neutral phosphate. As soon afterwards as possible we added 5 cc. of 0.1 per cent glycogen. The greatest care was taken not to introduce saliva into any of the reagents lest it digest

the glycogen. All solutions were kept at a given temperature in a thermostat bath.

While concanavalin A which has not been inactivated requires only 20 to 30 seconds to flocculate glycogen under the above conditions, reactivation at 18° may require as much as 42 minutes after complete inactivation. The curve in Fig. 2 shows the minutes required for reactivation after concanavalin A is allowed to stand with acid at room temperature for 48 hours. The inactivation was complete in this case. Total inactivation at 30° required about 120 minutes and at 25° about 300 minutes, as can be told by inspection of the curves in Fig. 1. Shaking greatly hastens the rate of flocculation of the concanavalin A-glycogen compound and the results vary as much as 100 per cent, depending

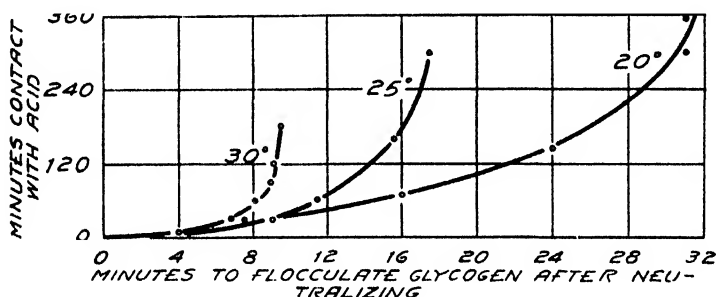


FIG. 1

upon whether the tests are shaken often or not at all and depending upon what one calls incipient flocculation. Hence it is unusual for two persons to obtain the same results.

We have found that when crystals of concanavalin A are dissolved in 0.1 N hydrochloric acid, placed in a collodion sack, and dialyzed for a day against several changes of distilled water, the material loses much of its ability to become reactivated upon being neutralized. This change caused by dialysis can be demonstrated by testing with glycogen as follows: Place 3 drops of 2 per cent acidified and dialyzed concanavalin A in a test-tube; add 1 cc. of distilled water and 8 drops of M sodium acetate to neutralize. Mix and allow to stand 15 minutes for reactivation, then add 5 cc. of 0.1 per cent glycogen, and mix. If the concanavalin A has become irreversibly inactivated, no precipitate will form for

nearly an hour. The irreversible inactivation of acidified con-canavalin A cannot be prevented by dialyzing it against sodium chloride instead of distilled water. It is not caused by contact with the collodion membrane, as can be shown by allowing con-canavalin A solution to stand in a beaker in contact with shredded collodion membrane.

We collected the outer liquid from dialyzing acidified con-canavalin A and concentrated it by boiling down. When some of this liquid was added to the neutralized, inactive concanavalin A, reactivation took place, as could be followed by the ability to precipitate glycogen and to agglutinate starch granules and ery-

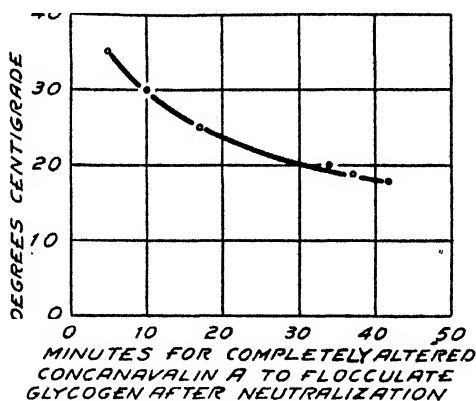


FIG. 2

throcytes. Complete reactivation required 5 to 30 minutes, depending upon the quantity of dialysate added, and was retarded if the glycogen was added first. Addition of dilute sodium hydroxide to the concentrated outer dialysis liquid precipitated most of the active material as a slightly colored gel. Spectroscopic analysis of the precipitate showed large amounts of calcium, manganese, and magnesium in addition to sodium, potassium, and traces of iron. Analysis of crude concanavalin A by the method of Willard and Greathouse (4) has shown 0.016 per cent of manganese. One recrystallization increased this to 0.021, two recrystallizations to 0.0226, and three recrystallizations to 0.0232 per cent. Air-dried jack bean meal contained 0.00195 per cent of manganese.

We have therefore tested various dilutions of solutions of the chlorides or sulfates of divalent metals with respect to their ability to reactivate acidified and dialyzed concanavalin A and find that many of them are effective. We have employed the procedure described above, in which 1 cc. of the metallic salt has been added in place of 1 cc. of distilled water. Arranged in order of their effectiveness the metals tested are

Ca, Mn, Zn, Mg, Co, Ni, Sr, Cu, Ba, Li, Na

In the middle of the series it is a matter of some difficulty to decide upon the order. A solution containing equal normalities of calcium, manganese, and magnesium is more effective than calcium alone. Indeed, a solution containing as little as 0.3×10^{-6} M Ca, Mn, and Mg will still produce a perceptible effect when 1 cc. is employed. Here the concentration of each metal is about 1 to 500 million. Thus it is seen that the divalent metals normally present in preparations of concanavalin A are the most effective among those tested, except that zinc, which is not ordinarily present, is more effective than magnesium.

Blank tests will ultimately give a small precipitate with glycogen. This may mean that dialysis has not removed all of the divalent metals originally present, that the reagents were not pure, or that sodium ions from the sodium acetate have some ability to produce reactivation.

It may be claimed by colloid chemists that the action of divalent ions, such as those of calcium, manganese, magnesium, etc., is due simply to their ability to precipitate by neutralizing charges on colloidal particles. We do not accept this view because reactivation by divalent metals requires time and because calcium ions are extremely effective, whereas barium ions have almost no action.

Concanavalin A which has been deprived of its manganese, calcium, and magnesium, is probably more soluble at pH 7.0 than unaltered concanavalin A. Its specific rotation is not changed, neither is its rate of migration in an electric field. In the absence of divalent metals concanavalin A does not crystallize well, and, if sufficiently concentrated, a considerable quantity of the protein will separate out in the amorphous state on standing.

When one adds salts of manganese, calcium, etc., the concanavalin A crystallizes readily. One observes that the better the crystallization the smaller the volume of precipitate. In one experiment we have noted the following order of effectiveness of metallic salts for promoting crystallization, the more effective metals being placed first.

Ca-Mn-Mg mixture, Mn, Ca, Mg, blank

This phenomenon can be connected with the observation made by Scott (5) that insulin does not crystallize unless it contains traces of certain metals, such as zinc, nickel, cobalt, or cadmium.

SUMMARY

Contact with dilute mineral acid causes concanavalin A to lose its ability to agglutinate erythrocytes and starch granules and to precipitate glycogen. After neutralization the ability of concanavalin A to agglutinate and to precipitate returns. The rates of inactivation and reactivation are hastened by rise of temperature.

Dialysis experiments show that the action of acid is to split off calcium, manganese, and magnesium. When salts of these metals are added to concanavalin A which has been irreversibly inactivated by treatment by acid, followed by dialysis, the activity of the concanavalin A is completely restored.

Concanavalin A which has been deprived of its calcium, manganese, and magnesium does not crystallize readily, whereas the addition of salts of these metals allows the protein to crystallize in a normal manner.

The findings described in this paper show that the ash of a protein is not necessarily to be looked on as impurity, but that proteins may be thought of as composed of an organic part united with one or more metals.

We wish to thank Mr. F. M. McNall for making a spectroscopic examination of the metals in the dialysate from concanavalin A.

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PURIFICATION OF THE ANTIHEMORRHAGIC VITAMIN BY DISTILLATION

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(Received for publication, June 24, 1936)

A method for the concentration of the antihemorrhagic vitamin from alfalfa has been described in a preceding paper.¹ It is the purpose of the present report to describe results obtained in an attempt to purify this vitamin further by distillation under a high vacuum.

An apparatus was set up with a Cenco Hyvac mechanical oil pump and two glass oil vapor diffusion pumps in series. These were connected to a mercury vacuum gage and to a distillation vessel. The distillation vessel consisted of a glass cylinder, inside of which was placed an upright water-cooled glass condenser with a flat, circular lower end. This end of the condenser was held at a distance of 3.5 cm. from the lower end of the glass cylinder, which was also closed and flattened. Heat was applied to the distillation vessel by means of an oil bath and electrical heating coils.

A concentrate prepared as described in the preceding paper¹ was dissolved in hexane and the solution placed in the distillation vessel. The solvent was then driven off by applying gentle heat and the pressure reduced by connection with an aspirator for several hours. The condenser was then inserted and the vessel evacuated by the mechanical and diffusion pumps to a pressure of approximately 10^{-6} mm. of mercury.

Warming the distillation vessel to a temperature of 40° caused a distillation of a colorless oily fraction, all of which passed over to the foot of the condenser at a temperature below 70°. This fraction was about 50 per cent of the crude vitamin concentrate.

Assays with chicks¹ revealed that the colorless oily fraction contained no detectable amount of the antihemorrhagic vitamin, while

¹ Almquist, H. J., *J. Biol. Chem.*, **114**, 241 (1936).

the non-distilled residue was adequate as a source of the antihemorrhagic vitamin at a level as low as 1 mg. per kilo of diet. This residue was about twice as potent as the original concentrate.

The residue was then distilled further and a fraction consisting of a yellow oil was obtained at a temperature of 120–140° during an interval of 2 hours. Assays revealed that this second distillate fraction contained the antihemorrhagic vitamin in somewhat greater concentration than did the non-distilled residue remaining after this fractionation. Further distillation of the residue produced more of the yellow oil fraction.

Another preparation of the vitamin concentrate was made from 6 kilos of alfalfa meal. 610 mg. of concentrate were obtained.

TABLE I
Occurrence of Hemorrhagic Symptoms in Chicks

Supplement	Level fed per kilo of diet	No. of chicks	Incidence of hemorrhagic symptoms in chicks at 14 days
	mg.		per cent
None.....		12	75
Colorless oil fraction.....	5	12	67
Yellow " ".....	0.5	12	0
" " ".....	1	12	0
" " ".....	2	12	0
" " ".....	3	12	0
Non-volatile residue.....	3	10	70

This was carefully distilled under a high vacuum into three fractions. The first was the colorless oil distilling at 40–60°. It amounted to 280 mg. or 46 per cent of the original concentrate. The second was a yellow viscous oil distilling at 120–145° in the amount of 230 mg. or 38 per cent. The residue consisted of a reddish material which had not distilled perceptibly at 145° after 4 hours. This residue contained most of the pigments of the original concentrate, probably carotenoids. It amounted to 100 mg. or 16 per cent of the concentrate.

Assays with chicks were conducted with these fractions (Table I). The first or colorless oil fraction gave no evidence of potency at 5 mg. per kilo of diet. The second fraction proved adequate at

0.5 mg. per kilo of diet, the lowest level at which it was tested. The residue was not adequate at a level of 3 mg. per kilo of diet and gave little evidence of containing any of the vitamin. Hemorrhagic symptoms occurred as soon when this residue was fed as when none of the vitamin was fed.

A second preparation of the antihemorrhagic vitamin, made as already described, has also proved adequate for the prevention of all hemorrhagic symptoms at a level of 0.5-mg. per kilo of diet.

It is evident that the distillation procedure brought about much further purification of the antihemorrhagic vitamin, since the second distillate was at least 4 times as potent as the original concentrate. Such purification is essential to further studies of this vitamin.

It is also evident that the requirement of this vitamin by the chick is not more than 0.5 mg. per kilo of diet or 0.5 microgram per gm. of diet.

ON PROTEOLYTIC ENZYMES

XI. THE SPECIFICITY OF THE ENZYME PAPAIN PEPTIDASE I

BY MAX BERGMANN, LEONIDAS ZERVAS,* AND
JOSEPH S. FRUTON

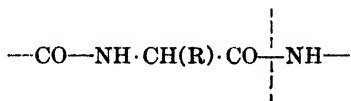
(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, July 8, 1936)

Papain Peptidase I was designated as that component enzyme of papain which splits hippurylamide, benzoylisoglutamine, and many other synthetic substrates and which is inhibited by phenylhydrazine (1-3). Papain Peptidase I occupies a singular position among the proteolytic enzymes for which synthetic substrates are known (4). It is the first enzyme of this group the specificity of which does not rigorously require the splitting of terminal peptide bonds, but operates instead in accordance with another more complicated principle.

Structural Specificity of Papain Peptidase I

Papain Peptidase I attacks only those substrates which contain two peptide bonds in the following arrangement.



I

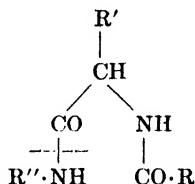
The polar character of this atomic group results in an inequality in the two peptide bonds. The enzyme is, in fact, able to split only one of the two, namely, the one indicated by the broken line.

In many membered polypeptides and proteins, adjacent peptide bonds, as shown by formula (I), are present in a manifold repetition. If such substrates are subjected to the action of Papain

* Fellow of the Rockefeller Foundation.

Peptidase I, then, as was shown by our first experiments, the enzyme selects particular peptide bonds from among those present for the splitting. The criteria which determine the nature of this selective process cannot be described by means of a simple chemical formulation. The basis of the nature of the selective process may be gleaned from the comparative enzymatic study of suitably chosen substrates. A number of such experiments are reported in this paper.

In the first place, there were studied a number of the simplest possible substrates of the general formula (II), in which R·CO was the benzoyl or carbobenzoxy group,¹ in order to determine the effect of variation of the substituents R' and R'' in formula (II) upon the velocity of splitting by Papain Peptidase I.



II

The simplest compounds of formula (II) are of course the substituted hippurylamides (R = C₆H₅; R' = H). Hippurylamide itself (R'' = H) is split quite rapidly by Papain Peptidase I. With our enzyme preparations, a splitting of nearly 100 per cent is obtained in 24 hours at 40°. Substituted hippurylamides, in which R'' is methyl, isoamyl, or phenyl, were split by the same enzyme solutions and under the same conditions only to a few per cent. Thus, neutral hydrocarbon residues, when acting as substituents in the amino group of hippurylamide, inhibit the splitting by Papain Peptidase I very strongly.

In order to extend the comparison to compounds having a

¹ We have repeatedly split benzoyl and carbobenzoxy derivatives of the same substance with Papain Peptidase I and have been unable to observe any marked difference in the velocity of splitting. It appears, therefore, permissible to compare the splitting by Papain Peptidase I of the benzoyl derivative with the carbobenzoxy derivative of another substance, as long as one compares only approximate reaction velocities in an extended series of experiments.

free carboxyl in the group R" (II), carbobenzoxyglycylglycine, carbobenzoxydiglycylglycine, and carbobenzoxytriglycylglycine were subjected to enzymatic hydrolysis. All three of these acyl peptides are split by Papain Peptidase I with the formation of carbobenzoxyglycine and the splitting proceeds more rapidly as the length of the peptide chain in R" is increased. It does not appear justifiable to interpret this finding as an indication of an inhibitory action of the free carboxyl group on the enzymatic hydrolysis. Upon passing successively from hippurylamide (III) to hippuryl methylamide (IV) and carbobenzoxyglycylglycine (V), *i.e.* upon the successive introduction of methyl and carboxyl groups, there results a marked inhibition of the enzymatic hydrolysis only after introduction of the methyl group but not following the carboxylation.

$C_6H_5 \cdot CO-NH \cdot CH_2 \cdot CO-NH_2$	}	Marked decrease in velocity of splitting
III		
$C_6H_5 \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_3$	}	No decrease in velocity of splitting
IV		
$C_6H_5 \cdot O \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_2 \cdot COOH$	}	Marked increase in velocity of splitting
V		
$C_6H_5 \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_2 \cdot CO-NC_6H_{10}$	}	
VI		

In order further to supplement this series, benzoylglycylglycine piperidide (VI) was synthesized and its enzymatic hydrolysis compared with that of carbobenzoxyglycylglycine. The two compounds differ in the fact that (V) contains a free carboxyl group, while (VI) has an acid piperidide group in the corresponding position. The piperidide is split much more rapidly by Papain Peptidase I than is the acid (V). However, in this comparison also, it would be misleading to attribute the increase in the velocity of splitting to the substitution of the carboxyl group. It would be worth while to investigate the replacement of the carboxyl by an acid amide group.

Experiments similar to those with the substituted hippurylamides were performed with derivatives of carbobenzoxyisoglutamine (carbobenzoxyglutamyl- α -amide (VII)).

TABLE I

*Hydrolysis of Substituted Acylamino Acid Amides by HCN-Papain
Peptidase I*

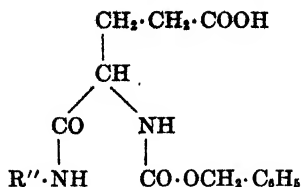
The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Substrate	Hydrolysis							Isolated products
	1 hr.	2 hrs.	3 hrs.	4 hrs.	24 hrs.	48 hrs.	144 hrs.	
Gelatin.....	0.79				1.32			Hippuric acid
Hippurylamide				0.42	0.85			
Hippuryl methylamide..				0.06	0.15	0.18		
Hippurylisoamylamide*.				0.02	0.04			
Hippurylanilide*.....				0.01	0.02			
Hippurylglycine piper- idide.....				0.60	0.95			Hippuric acid (80% theory)
Carbobenzoxylglycylgly- cine					0.25			Glycine
Carbobenzoxylglycylgly- cine.....					0.48			Glycylglycine
Carbobenzoxylglycylgly- cine					0.85			Diglycylgly- cine
Gelatin.....	0.92				1.83			
Carbobenzoxylglutamyl- α-amide†.....	0.39				0.90			
“ ‡		0.65		0.83	0.88			
Carbobenzoxylglutamyl- α-methylamide†.....			0.04		0.34			
“ ‡		0.05		0.07	0.42			
Carbobenzoxylglutamyl- α-isoamylamide.....			0.18		0.77			
Gelatin.....	0.92				1.44			
Carbobenzoxylglutamyl- α-glycine					0.30	0.48	0.90	
								Carbobenzoxyl- glutamic acid

* Did not go into solution at start of reaction.

† 1 equivalent of 0.5 N ammonia was added.

‡ 1 equivalent of 0.5 N sodium acetate was added.



VII

Carbobenzoyisoglutamine^a, like all the other test substances mentioned before, is based on the general formula (II). It differs from the hippurylamides in the fact that it contains as R' an acidic group with a free carboxyl group. The enzymatic cleavage of carbobenzoyisoglutamine was compared with the splitting of its derivatives which have a methyl or amyl group as R''. It will be noted from Table I that these substitutions cause a decrease in the velocity of the enzymatic hydrolysis, the methyl group being a stronger inhibitor than the isoamyl group. The inhibition becomes particularly clear if, for the purposes of the comparison, one considers the initial hours of the reaction in which the amounts of the inhibitory reaction products are still quite small. Furthermore, carbobenzoyglutamylglycine, which has a carboxyl group in R'', was drawn into the comparative study and showed that no marked inhibition by the carboxyl group could be observed. A relationship similar to that for the hippurylamides is thus found here, the only difference being that the derivatives of carbobenzoyisoglutamine mentioned above are split much more rapidly than are the corresponding derivatives of hippurylamide. This difference may be attributed to the presence of the side chain $\text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$ in the isoglutamine derivatives.

It was to be expected from experiences with other proteolytic enzymes that the action of Papain Peptidase I would be influenced by structural details of the substrate molecule. In the case of papain, however, what is surprising is the magnitude of this effect, since a very slight change, such as substitution of neutral hydrocarbon groups for a hydrogen group, produces a great difference in splitting. It is further noteworthy that such effects are not confined to those parts of the substrate molecule immediately adjacent to the peptide bond which is to be split. This great sensitivity of the enzyme toward structural influences and the

TABLE II

Hydrolysis of Acylated Peptides by HCN-Papain Peptidase I

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Phenylhydrazine per cc. of test solution	Substrate	Hydrolysis				Isolation of products
		2 hrs.	6 hrs.	24 hrs.	144 hrs.	
<i>mM</i>						
	Gelatin	0.92		1.44	..	
	Carbobenzoxy-L-glutamylglycine*		0.07	0.30	0.90	Carbobenzoxy-glutamic acid (70 % theory)
	Carbobenzoxy-L-glutamylglycylglycine*		0.38	0.96	1.58	Carbobenzoxy-glutamylglycine (55 % theory after 24 hrs.)
		1 hr.	5 hrs.	24 hrs.		
0.0025	Gelatin	0.81				
	"	0.47				
	Carbobenzoxy-glycyl-L-glutamylglycine		0.37	0.83		
0.0025	"		-0.01	0.02		
	Carbobenzoxy-L-glutamylglycylglycine			0.80		
0.0025	"			0.04		
		1 hr.	2½ hrs.	7 hrs.	4 hrs.	
	Gelatin	0.87			1.80	
	Benzoyl-L-leucyl-L-leucylglycine		0.50	1.03	1.20	Leucylglycine as carbobenzoxy derivative (67 % after 7 hrs.)

* 1 equivalent of 0.1 N NaOH was added; 1 equivalent of 0.5 N sodium acetate was added.

fact that the enzyme is not restricted to a certain fixed point of attack in the polypeptide molecule often lead to the result that the enzyme splits two quite similar substrates at different linkages in the molecule. It is therefore essential to investigate a greater number of substrates for Papain Peptidase I with respect to reaction velocity and point of attack. Only in this manner can one hope gradually to gain an insight into the rules of specificity which determine the details of the enzyme action.

TABLE III

Carbobenzoxyglycyl		glycine
" "		glycylglycine
"		glycylglycylglycine
Benzoylglycyl		<i>l</i> -leucylglycine
"		glycyl- <i>l</i> -leucylglycine
Carbobenzoxyglycyl		glycylglycyl <i>l</i> -leucylglycine
Carbobenzoxy- <i>l</i> -leucylglycyl		glycine
Benzoyl- <i>l</i> -leucylglycyl		glycine
Benzoyl- <i>l</i> -leucyl		<i>l</i> -leucylglycine
Carbobenzoxyglycyl		<i>l</i> -glutamylamide
"		<i>l</i> -glutamylglycine
Carbobenzoxy- <i>l</i> -glutamyl		glycine
"		² glycyl ¹ glycine
Benzoyl- <i>l</i> -lysine		amide
Benzoylglycyl		<i>l</i> -lysylglycine
"		² carbobenzoxo- <i>l</i> -lysyl ¹ glycine

The favored point of splitting is indicated by the numeral 1; the second point, by the numeral 2.

Table II shows the enzymatic hydrolyses of several newer substrates. These results are to be employed as a basis for further discussion.

In Table III there are collected a number of substrates for Papain Peptidase I. Each of these substrates offers the enzyme the choice of several possible points of attack. The actual position of splitting is indicated in Table III by vertical lines. In several substrates the splitting takes place at two linkages with a widely differing speed or in succession. The favored point of splitting is indicated by the numeral 1; the second point, by the numeral 2.

It is evident from Table III that Papain Peptidase I is not

limited in its action to the peptide linkages between particular amino acids. The enzyme is, on the contrary, able to hydrolyze the most varied of peptide bonds regardless of the presence of neutral, acidic, or basic amino acids on the carbonyl or imino side of the split linkage. However, when the enzyme acts on a substrate which contains several peptide linkages, it attacks them with widely different velocities. The splitting does not occur haphazardly at all the peptide linkages, but only certain ones are preferred. The characteristics of this relative specificity in the case of acylated peptides may be stated as follows:

The acylamino group of acylated peptides directs the splitting by Papain Peptidase I to the peptide linkage immediately adjacent to the acylamino group.

If, in addition to peptide bonds with glycine-carbonyl, an acylated polypeptide contains peptide linkages with leucine-, glutamic acid-, or lysine-carbonyl, then papain peptidase splits the peptide bond with the glycine-carbonyl. It appears, furthermore, that in all of the cases studied, the directing influence of the glycine-carbonyl is stronger than that of the acylamino group. Thus, if the peptide bond adjacent to the acylamino group has no glycine-carbonyl, but another peptide bond of the molecule does contain one, then the point of splitting is shifted away from its proximity to the acylamino group.

It might be thought that perhaps this preference for glycine was connected with its low molecular weight; *i.e.*, that papain peptidase chose those peptide bonds the carbonyl of which belonged to the amino acid with the lowest molecular weight. However, from the last two examples in Table III it appears that this is not the case. In benzoylglycyl-*L*-lysylglycine the enzyme splits the peptide bond which is adjacent to the acylamino group and which also contains a glycine-carbonyl. In benzoylglycyl- ϵ -carbobenzoxy-*L*-lysylglycine, however, that peptide bond is split which contains the carbonyl of the lysine (the amino acid with the highest molecular weight). The introduction of an acid amide group at the end of the long side chain of lysine has so powerful an effect that the combined influence of the acylamino group and the glycine residue is overcome.

It has hitherto never been found that the lysine residues in proteins are acylated. On the other hand, it is known that the

side groups of glutamic and aspartic acids are in the amide form. One might expect, in connection with the experiences mentioned above, that such acid amide groups in the side groups of proteins play a rôle in directing the enzymatic hydrolysis.

Hydrolysis of Free Polypeptides by Papain Peptidase I

The hitherto mentioned experiments on the specificity of Papain Peptidase I are concerned with the splitting of acylated peptides.

TABLE IV

Hydrolysis of Polypeptides by HCN-Papain Peptidase I

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates. The test solution was kept at pH 5 and 40° in all cases.

Phenyl- hydrazine per ml. of test solution	Substrates	Hydrolysis						
		1 hr.	1 day	2 days	3 days	4 days	5 days	6 days
<i>mM</i>								
	Gelatin	1.00	1.86					
0.0025	"	0.54	1.16					
0.005	"	0.45	0.94					
	Diglycyl-L-leucylglycine			0.47			0.73	
	"		0.21	0.42	-	0.68		0.75
	Triglycyl-L-leucylglycine		0.52	0.86	1.02			1.32
	"		0.58	0.91	1.07	1.12		1.46
0.0025	"				0.08*			
0.005	"				0.11*			

* The presence of phenylhydrazine caused the test solution to turn brownish yellow; these titration values are therefore probably too high because of overtitration.

The action of the enzyme is, however, not confined to acylated peptides. It was found that papain splits free peptides as well (5). In Table IV are given the rates of splitting of diglycyl-L-leucylglycine and of triglycyl-L-leucylglycine. It will be noted that the enzyme action on the pentapeptide involves the splitting of more than one peptide linkage. It was found further that the splitting of the two polypeptides which were studied is inhibited completely by phenylhydrazine. There can therefore be no doubt

that the hydrolysis of these free polypeptides is performed by the same enzyme as the one which splits the acylated peptides, and which is similarly inhibited by phenylhydrazine; namely, Papain Peptidase I (6). Bergmann and Ross have shown recently that the splitting of gelatin by activated papain is strongly inhibited by phenylhydrazine, and concluded from this finding that Papain Peptidase I participates significantly in the papain digestion of gelatin. This conclusion is supported by the finding that the enzyme attacks free polypeptides. Furthermore, the earlier observation of Willstätter and Grassmann (7) that papain is able to split the tripeptide, leucylglycylglycine, finds a wider significance.

One of the immediate problems is to investigate a large number of polypeptides of various amino acids in their behavior toward Papain Peptidase I, in order to collect further material regarding

TABLE V

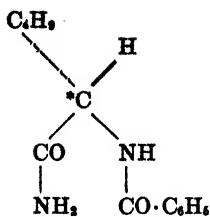
Hydrolysis of d- and l-Benzoylleucineamide at 40° and pH 5

	24 hrs.	48 hrs.
	per cent	per cent
<i>l</i> -Amide.....	79	84
<i>d</i> -Amide.....	0	0

the specificity of the enzyme. It is not improbable that the enzyme acts differently toward free and acylated peptides.

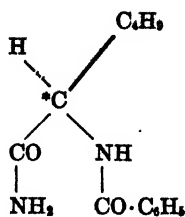
Antipodal Specificity of Papain Peptidase I

The discovery of synthetic substrates for Papain Peptidase I made it possible to study the antipodal specificity of the enzyme. The simplest substrates for such experiments are the two antipodes of benzoylleucineamide. If the formulas of the two antipodes are reproduced in spatial projection, the C.N.C.C.N— chain, the essential grouping of peptide bonds which both antipodes contain, is to be visualized as lying in the plane of the paper (VIII and IX). In this chain the asymmetric carbon lies between the two peptide bonds. It is indicated by an asterisk. The two substituents of the asymmetric carbon atom, H and C₄H₉, do not lie in the plane of the paper but in front of, or behind it.



VIII

l-Benzoylleucineamide. (The H at the asymmetric carbon atom is to be visualized as being in front of the plane of the paper, the C₆H₅ behind the plane)



IX

d-Benzoylleucineamide. (The H at the asymmetric carbon atom is to be visualized as being behind the plane of the paper, the C₆H₅ in front of the plane)

It will be seen from Table V that Papain Peptidase I splits only one of the antipodes; namely, the one derived from the natural *l*-leucine. Papain Peptidase I therefore shows antipodal specificity and is restricted to the natural *l* configuration of the substrates just as are dipeptidase and the other known digestive enzymes.

In order to explain the antipodal specificity of certain enzymes, Emil Fischer made the assumption that these enzymes were themselves built asymmetrically. This generally accepted concept may be more closely defined as follows: If an enzyme catalyzes only one of two antipodes, then it must contain at least three different atoms or atomic groups which are fixed in space with respect to one another, these groups entering during the catalysis into relation with a similar number of different atoms or atomic groups of the substrate.² Through this polyaffinity relationship, the active groups of the enzyme force the active groups of the substrate into a definite spatial arrangement with respect to each other and to the enzyme. One might imagine in the enzyme-substrate combination a plane formed by the active groups of the enzyme (binding plane of the enzyme) and a plane formed by the active groups of the substrate (binding plane of the substrate). Then the requirements for the formation of an enzyme-substrate combination and for the appearance of antipodal specificity may be formulated as follows: During enzymatic catalysis the binding

² The peptide bond is considered double because of its dipole character.

plane of an enzyme with polyaffinity approaches within a few Ångström units of the binding plane of the substrate. If the substrate is so constructed that one or more large atomic groups³ jut out from the binding plane into the space between the binding planes of substrate and enzyme, then the approach of the binding plane of the enzyme toward the binding plane of the substrate is prevented. The catalysis does not occur as a result of steric hindrance.

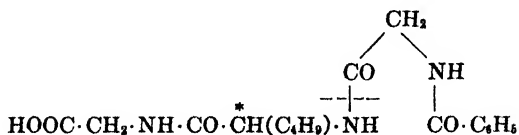
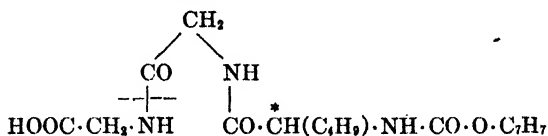
Antipodal specificity has been found for all the proteolytic enzymes the specificity requirements of which could be investigated with substrates of known structure, and in all these cases it was possible to confirm the polyaffinity relationship. If one allows such an enzyme to act on the two antipodes of a substrate and determines which antipode is split, it is possible to decide which spatial arrangement of the active groups of the substrate is required by the enzyme and from which side the enzyme approaches the binding plane of the substrate during catalysis. The spatial picture obtained in this way allows one to predict the enzymatic behavior of the antipodes of all possible substrates of the same enzyme, always assuming that the center of antipodal asymmetry lies within the binding plane of the substrate. For Papain Peptidase I it may be concluded from the splitting of benzoyl-*l*-leucineamide that the enzyme splits only that antipode of an optically active compound with a large R' in which the arrangement $\text{NH} \cdot \text{CO} \cdot \text{CH}(\text{R}') \cdot \text{NH} \cdot \text{CO}$ is present in a clockwise order as viewed from the binding plane of the enzyme, and in which the H of the CHR' group points toward the enzyme. In the case of benzoyl-*d*-leucineamide and other derivatives of *d*-amino acids, these two requirements are not fulfilled simultaneously; these compounds cannot therefore be split by Papain Peptidase I.

Von Euler and Josephson have set up a two affinity theory of enzyme action (9). This theory requires two different groups in the enzyme, one of which is responsible for the combination with

³ To explain what is meant by large atomic groups, it might be recalled that with dipeptidase a methyl group in the prohibited position markedly slows down the catalysis, but that an isobutyl group prevents any splitting whatever in the case of dipeptidase (8) as well as of Papain Peptidase I. From these facts, it is concluded that the necessary approach must be within a distance of several Ångström units.

the substrate, while the other performs the splitting. We extend the theory of von Euler and Josephson in replacing the two affinity concept by a polyaffinity relationship, and assume a fixed relative position for the active groups in the enzyme. This extension makes it possible to explain antipodal specificity. As far as we know, this is the first case in which the specificity of a group of enzymes can be clarified with the aid of a simple hypothesis. It is possible that the antipodal specificity of the serological reactions discovered by Landsteiner and van der Scheer (10) may be explained with the aid of similar considerations (polyaffinity and spatial fixity of active groups in the antibodies).

The discussion of antipodal specificity has concerned itself until now with the asymmetry of those carbon atoms which lie between the active groups of the substrate; *i.e.*, within the binding plane of the substrate. For the substrates of Papain Peptidase I, there is involved only a single carbon atom of this kind; namely, the one lying between the two essential adjacent peptide bonds. It should be pointed out that two other cases of a different type of antipodal specificity have been observed for Papain Peptidase I. The substrates involved are the antipodes of carbobenzoxy-leucylglycylglycine (X) and benzoyl-glycylleucylglycine (XI).



XI

In both formulæ, the peptide bond which is split is designated by a broken line and the two peptide bonds which determine the binding plane of the substrate are drawn as in all the previous formulæ of this type. It is evident that in both substrates the asymmetric carbon atoms lie outside the binding planes of the respective sub-

strates. However, as was reported earlier (1), there occurs antipodal specificity. Perhaps one should conclude from these examples that during the combination with the enzyme not only is the binding plane forced into a definite spatial position with respect to papain peptidase, but neighboring parts of the substrate molecule are fixed as well.

EXPERIMENTAL

Carbobenzoxylglutamylglycylglycine

Carbobenzoxylglutamylglycylglycine Ethyl Ester—To 7.4 gm. of carbobenzoxylglutamylglycine ethyl ester (1) in 10 cc. of absolute alcohol there were added 3 cc. of hydrazine hydrate, and the reaction mixture was left at room temperature for 24 hours. On addition of anhydrous ether, a syrupy precipitate resulted. After being washed several times with ether, the syrup was dissolved in 100 cc. of ice-cold water, covered with ether-ethyl acetate (1:1), and treated, at 0° with shaking, with 40 cc. of cold 5 N hydrochloric acid and an aqueous solution of 1.7 gm. of sodium nitrite added in several portions within 3 to 4 minutes. After washing the ether-ethyl acetate layer with water until the acid reaction with Congo red had disappeared, the solution was passed through a dry filter, dried briefly over Na_2SO_4 , and mixed with an ethereal solution of glycine ethyl ester (prepared from 15 gm. of hydrochloride). The mixture was evaporated under diminished pressure, leaving a syrup which was heated for 3 hours at 30°, then for 5 minutes at 80°, dissolved in cold water, and acidified with 5 N hydrochloric acid to Congo red. The syrup which was precipitated crystallized on standing. On recrystallization from hot alcohol, followed by recrystallization from ethyl acetate, there were obtained needles with a melting point of 139–140°. Yield, 2.8 gm.

$\text{C}_{19}\text{H}_{25}\text{O}_5\text{N}_3$ (423.2). Calculated, N 9.9; found, N 10.0 (Kjeldahl)

The same substance is obtained by coupling carbobenzoxylglutamyl anhydride with glycylglycine ethyl ester in ethyl acetate solution. There are formed two isomers (α - and γ -peptides); the isolation of the required α -peptide in a pure state is difficult, giving only a small yield.

Carbobenzoxylglutamylglycylglycine—2.8 gm. of the above ester were dissolved in 15 cc. of NaOH, and after 30 minutes acidified

to Congo red with 5 N hydrochloric acid. The reaction mixture was concentrated at 40°, under diminished pressure, to about 4 cc. and the carbobenzoxytripeptide extracted with a large volume of ethyl acetate. The ethyl acetate solution was washed with 2 cc. of water, evaporated down, and the residue dissolved in dry acetone. The acetone solution was evaporated, leaving a syrup which when treated with a small volume of dry ethyl acetate at 0° yielded 1.6 gm. of small plates which melted at 142°.

$C_{17}H_{21}O_5N_3$ (395.2). Calculated, N 10.6; found, N 10.6 (Kjeldahl)

In the splitting of the carbobenzoxytripeptide by papain, more than one peptide linkage was hydrolyzed. A portion of the reaction mixture was worked up after 24 hours, at which time the increase in carboxyl represented about 1 mole. It was concentrated under diminished pressure, acidified, extracted with a large volume of ethyl acetate, the extract evaporated down, and the residue brought to crystallization with ether. Carbobenzoxyglutamylglycine was obtained in 55 per cent yield. M.p., 143°. The mixed melting point with a preparation made according to Grassmann and Schneider (11) showed no depression.

$C_{15}H_{19}O_7N_2$ (338.2). Calculated, N 8.3; found, N 8.3 (Kjeldahl)

l-Leucyl-l-Leucylglycine

Carbobenzoxy-l-Leucine—To the solution of 13.1 gm. of *l*-leucine in 50 cc. of 2 N NaOH there were added, at 0° with shaking, 17 gm. of carbobenzoxy chloride and 60 cc. of 2 N NaOH in several portions. The syrup obtained on acidifying was taken up in ether and the ethereal solution extracted with potassium bicarbonate solution. The bicarbonate solution was acidified, the carbobenzoxy-leucine again taken up in ether, and the ether evaporated off. Yield, 20 gm. of syrup.

Carbobenzoxy-l-Leucyl-l-Leucylglycine Methyl Ester—6 gm. of carbobenzoxy-*l*-leucylglycine (1) were dissolved in methanol containing 1 mole of aqueous hydrochloric acid and hydrogenated in the presence of palladium, the solution was evaporated down, and the dipeptide esterified as usual at 0° with methanol-hydrogen chloride. The free ester was liberated with potassium carbonate and taken up in ethyl acetate.

7 gm. of syrupy carbobenzoxy-*l*-leucine (dried over P_2O_5) were dissolved in 30 cc. of dry ether and treated as usual with 7 gm. of PCl_5 at 0° . The reaction mixture was filtered, diluted with 70 cc. of ether, washed three times with ice-cold water in a separatory funnel, passed through a dry filter, dried quickly over Na_2SO_4 , and added at 0° to the above solution of leucylglycine ester. The mixture was shaken with about 20 cc. of saturated potassium bicarbonate solution, the upper layer successively washed with hydrochloric acid, bicarbonate, and water, dried, and evaporated down. The resulting syrup was treated with ether-petroleum ether, yielding small prisms after standing overnight. The crystals were transferred to the filter with a small amount of ether. M.p., 108° . Yield, 3.6 to 4.0 gm.

$C_{23}H_{31}O_6N_3$ (449.3). Calculated, N 9.4; found, N 9.4 (Kjeldahl)

l-Leucyl-*l*-Leucylglycine—3 gm. of the above ester were dissolved in a mixture of methanol and 1.1 mole of 2 *N* NaOH. After 20 minutes the solution was acidified and the methanol evaporated off under diminished pressure. The resulting syrup was taken up in ethyl acetate-ether (1:1) and extracted with potassium bicarbonate. The bicarbonate extract was acidified and the substance again taken up in ethyl acetate, which was then evaporated off. The residue was dissolved in water-methanol containing 0.5 cc. of glacial acetic acid, and hydrogenated catalytically. The reaction mixture was evaporated down and the resulting crystals transferred to the filter with alcohol. Yield, 1.3 gm.

$C_{14}H_{27}O_4N_3$.	Calculated.	C 55.8, H 9.0, N 13.9
301.2	Found.	" 55.8, " 9.2, " 14.0 (Dumas)

Benzoyl-l-Leucyl-l-Leucylglycine—1 gm. of the tripeptide was suspended in a mixture of 10 cc. of half saturated potassium bicarbonate solution and treated at 0° with 0.6 cc. of benzoyl chloride in several portions over a period of 30 minutes. The syrup obtained on acidifying was treated with hot water and allowed to stand overnight. The substance was then dissolved in acetone, water added to incipient precipitation, and enough acetone added to dissolve. On standing at room temperature, 1.1 gm. of needles were obtained, which on recrystallization (as above) melted at 161° . The air-dried substance was analyzed.

$C_{21}H_{27}O_5N_3 \cdot \frac{1}{2}H_2O$. Calculated. C 60.9, H 7.9, N 10.1, H_2O 2.2
414.2 Found. " 60.9, " 7.6, " 10.1 (Dumas), " 1.7

The splitting of the benzoyl tripeptide by papain exceeded the hydrolysis of one peptide linkage. As soon as the splitting corresponded to the splitting of one peptide bond, a portion of the solution was concentrated under diminished pressure, acidified with hydrochloric acid, and extracted with ether. The aqueous portion was then made alkaline, carbobenzoxyolated, again acidified, and extracted with ether. On evaporating the ether, there was obtained carbobenzoxy-leucylglycine in 67 per cent yield. M.p. and mixed m.p., 113°.

Benzoylglycylglycine Piperidide

Carbobenzoxyglycyl Piperidide—To an ice-cooled, aqueous piperidine solution (about 5 moles of amine) there was added, with shaking, 1 mole of carbobenzoxyglycyl chloride in portions. After a short interval, the piperidide separated out in a 60 per cent yield (calculated for the chloride). On recrystallization from ether-petroleum ether, there were obtained needles. M.p., 78°

$C_{18}H_{20}O_3N_2$ (276.2). Calculated, N 10.2; found, N 10.5 (Dumas)

Benzoylglycylglycine Piperidide—Carbobenzoxyglycyl piperidide (1 mole) was dissolved in methanol and 2 moles of glacial acetic acid and hydrogenated catalytically. The solution was evaporated down, the residue dissolved in cold bicarbonate solution, and treated at 0° with 1 mole of hippuryl chloride in several portions. The chloride slowly went into solution while the piperidide separated out. Yield, 40 per cent. On recrystallization from aqueous bicarbonate solution and ethyl acetate there were obtained needles which melted at 134°.

$C_{18}H_{21}O_3N_2$ (303.2). Calculated, N 13.8; found, N 13.7 (Dumas)

Hippurylanilide—This substance was prepared from hippuryl chloride and aniline-water. M.p., 214°.

$C_{15}H_{17}O_2N_2$ (254.1). Calculated, N 11.0; found, N 10.8 (Kjeldahl)

Hippurylisoamylamide—This substance was prepared from hippuryl chloride and isoamylamine-water. M.p., 98°.

$C_{14}H_{20}O_2N_2$ (248.1). Calculated. N 11.3; found, N 11.0 (Kjeldahl)

Carbobenzoxy-l-Glutamyl Methylamide—4 gm. of carbobenzoxy-*l*-glutamyl anhydride were added within 5 minutes in portions to 7 cc. of ice-cooled 33 per cent aqueous methylamine solution. After 15 minutes, the reaction mixture was acidified, yielding a syrup which crystallized on scratching. The crystals (needles) were recrystallized from methanol-water. Yield, 2 gm. M.p., 178°.

$C_{14}H_{18}O_2N_2$ (294.1). Calculated, N 9.5; found, N 9.5 (Kjeldahl)

Carbobenzoxy-l-Glutamylisoamylamide—This substance was prepared in a similar manner as the methylamide. On recrystallization from methanol-water and ether, the melting point was 135°.

$C_{14}H_{20}O_2N_2$ (350.2). Calculated, N 8.0; found, N 8.3 (Dumas)

Diglycyl-l-Leucylglycine—This substance was prepared as described in (1).

Triglycyl-l-Leucylglycine—This substance was prepared as described in (1).

Benzoyl-l-Leucineamide—3 gm. of *l*-leucine ethyl ester hydrochloride were converted to the free ester in ether solution, which was then allowed to react with 2.2 cc. of benzoyl chloride in ethyl acetate and 16 cc. of a 10 per cent sodium carbonate solution. The ether layer was washed with water, bicarbonate, and dilute hydrochloric acid and evaporated down, yielding crystals which were allowed to react with gaseous ammonia in absolute alcohol for 3 days. The solution was then evaporated down, yielding a syrup which crystallized upon treatment with water. Upon recrystallization from ethyl acetate the substance melted at 187°. Yield, 2 gm.

$C_{13}H_{18}O_2N_2$. Calculated, C 66.7, H 7.6, N 11.8

238.2

Found. " 66.6, " 7.6, " 11.7 (Dumas)

$[\alpha]_D^{25} = -6.4^\circ$ (2.5 per cent in alcohol)

From the enzymatic hydrolysis to 84 per cent of 120 mg. of this substance there were isolated, in the usual manner, 100 mg. of a product melting at 105°.

Benzoyl-d-Leucineamide—This substance was prepared in the same manner as the *l* form. M.p. 187°.

$C_{13}H_{15}O_2N_2$.	Calculated.	C 66.7, H 7.6, N 11.8
238.2	Found.	" 66.5, " 7.8, " 12.0 (Dumas)
		$[\alpha]_D^{25} = +6.4^\circ$ (2.5 per cent in alcohol)

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ON THE ORIGIN OF URINARY CREATININE

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In 1922 Behre and Benedict reported work on the basis of which they came to the conclusion that creatinine does not occur in normal blood in detectable amounts. They did not fail to point out in their discussion that this finding has to be reconciled in some way with the appreciable amounts of creatinine excreted daily in the urine. In this connection they suggested two alternative mechanisms: (a) either the kidney is able to concentrate to an extreme degree such traces of creatinine as may be present in the blood beyond the possibility of accurate differentiation by the procedures used, or (b) the kidney must be able to manufacture creatinine from some precursor substance.

Regarding the first assumption it may be recalled that Van Slyke, Hiller, and Miller (1935) showed that in the dog ingested creatinine is excreted with exactly the same "clearance" and exactly the same "extraction percentage" as ferrocyanide and inulin. Since there exists considerable independent evidence that the latter two substances are excreted by glomerular filtration exclusively, creatinine (the preexisting as well as the super-imposed) should be excreted by this same mechanism.

The extraction percentage for creatinine found by these authors is approximately 20 per cent. This means that out of every mg. present in whole blood, only 20 per cent of the quantity present in the plasma can be excreted by the kidney in one passage, or, assuming that the plasma volume is approximately 55 per cent of the total, only $\frac{1}{5} \times 55 = 11$ per cent of the total amount present can be excreted in one passage through the kidney. Thus, only one-ninth of the "traces" which Behre and Benedict would

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be inclined to accept as existing in normal blood would be available for excretion, and this small quantity would certainly not be enough to explain the daily creatinine excretion.

Therefore the alternative assumption, *viz.* the formation of creatinine by the same organ which brings about its excretion, seems well worth considering. Incidentally the precursor substance, probably easily converted into creatinine, might also be responsible for part or all of the small amounts of creatinine isolated from blood filtrates and ultrafiltrates shaken with various adsorbents or saturated with picric acid. Gaebler (1930), for instance, found that although a blood filtrate may be shaken with kaolin without appreciably changing the color produced on the addition of alkaline picrate (a procedure extensively practised by Behre and Benedict), creatinine could be isolated and identified from subsequent washings of the kaolin.

To obtain some insight into the mechanism of renal excretion of creatinine, it was felt that possibly accurate comparison of the concentrations of apparent creatinine (or "chromogenic material;" this neutral name was proposed by Behre and Benedict for the material responsible for the Jaffe reaction in blood filtrates) in arterial and renal venous blood might throw some light on this confusing problem. If the kidney were manufacturing creatinine from non-chromogenic material, it was to be expected that the level in the renal vein should either be the same as that in the artery or possibly (in view of Nash and Benedict's (1921) experience with ammonia) be increased. If, however, chromogenic material were the direct source of urinary creatinine, its concentration in the vein should be found decreased as compared with the arterial level and this decrease should be of an order of magnitude such as might be expected from the creatinine excretion per minute and the rate of blood flow through the kidney.

At this point it may be worth while to consider whether any such differences between arterial and renal venous creatinine content, if present, might be large enough to be determined with a reasonable degree of accuracy. If we assume that part of the chromogenic material of the blood is removed by the kidney and excreted as creatinine, a tentative calculation of the resultant drop in the creatinine content of the blood can be made as follows: (1) the average creatinine excretion of a dog on a diet containing

no creatine or creatinine may be estimated (according to the data of Benedict and Osterberg (1923)) to be approximately 24 mg. per kilo of body weight per 24 hours, or $\frac{1}{80}$ mg. per minute per kilo of body weight; (2) the combined kidneys of a dog weigh approximately $\frac{1}{180}$ of the total body weight (Ellenberger and Baum, 1891) and the average perfusion of the kidney with blood may be put at 2.5 cc. per gm. of kidney per minute (Janssen, 1929) or $2.5 \times 1000/160 =$ about 15 cc. per minute per kilo of body weight. Thus, the $\frac{1}{80}$ mg. of creatinine should have been removed from a volume of 15 cc. of blood, indicating that under the

TABLE I

Showing Quantitative Recovery of Creatinine Added to Blood

All figures are expressed in mg. per 100 cc. of blood (thus, mg. per 1000 cc. of the 1:10 tungstomolybdic acid filtrate).

Apparent creatinine (1)	Creatinine added (2)	Same blood as in (1) after addition of creatinine (3)	Quantity of creatinine recovered (4)	Difference between creatinine added and creatinine recovered (5)
1.45	0.25	1.69	0.24	-0.01
1.61	0.25	1.87	0.26	+0.01
1.44	0.20	1.66	0.22	+0.02
1.43	0.20	1.63	0.20	
1.47	0.15	1.63	0.16	+0.01
1.41	0.15	1.56	0.15	
1.43	0.10	1.54	0.11	+0.01
1.56	0.10	1.65	0.09	-0.01

assumption outlined above a drop in creatinine content of approximately 0.11 mg. per 100 cc. of blood should be expected.

A difference of such an order of magnitude represents more than 5 times the maximum error which Goudsmit and Summerson (1935) met with in recovering creatinine, using the variable layer photoelectric comparison photometer as a measuring instrument. Moreover, pure creatinine added to blood (see Table I) has been recovered satisfactorily with a technique involving the use of a 1:10 tungstomolybdic acid filtrate (Benedict, 1931) and in accordance with the procedure for the comparative determination of creatinine outlined later in this article.

EXPERIMENTAL

It was planned to collect samples of renal venous and arterial blood and to analyze these samples for apparent creatinine. At the same time the urine, formed in a period corresponding with and representative for the time at which the samples of blood were to be drawn, was to be collected and analyzed for its creatinine content. In addition urea was to be determined on all samples of blood and urine in order to obtain data on the excretion of another substance naturally occurring in the body.

The experiments to be described below have been performed on dogs exclusively. The animals have been kept on different diets (see Table II, Column 1) for various lengths of time. No food, however, was administered 18 hours before the beginning of each experiment; water was allowed freely. The animals were anesthetized by intraperitoneal injection of amytal, 55 mg. per kilo of body weight, the anesthetic having been dissolved by the addition of a suitable quantity of sodium hydroxide. The left femoral artery was fitted with a cannula in order to allow the subsequent collection of arterial blood samples; likewise providing the left jugular vein with a cannula afforded means for later intravenous administration of fluids.

For the collection of representative samples of renal venous blood a simple method was devised¹ which may be described as follows: a bayonet-shaped glass catheter was inserted into the inferior vena cava, after ligation of this vein approximately 10 cm. distal to the mouth of the renal veins, followed by ligation of all other vessels draining into the vena cava between the point of insertion of the catheter and the renal veins. After the catheter had been fixed in place, the abdominal cavity was closed by the suture of its muscular and cutaneous walls, leaving one end of the catheter accessible.

Since under the conditions of the experiment a satisfactory volume of urine could hardly be expected, it was deemed desirable to administer additional quantities of fluids. This was effected by the slow (about 5 cc. per minute) intravenous injection of the usual mammalian Ringer's solution. In the case of the last three experiments this solution was modified so as to contain 0.83

¹ I am greatly indebted to Dr. Dayton J. Edwards for his helpful co-operation and many valuable suggestions during the course of this work.

per cent sodium chloride instead of 0.92 per cent, which (as can be seen in Table II, Column 3) has considerably increased the rate of urinary flow. Thus the dogs maintained a highly satisfactory excretion of urine and, as will be seen from the data to be presented, in most cases fail to show appreciable signs of impaired kidney function.

In order to delay thrombus formation caused by the continuous contact of the blood with a foreign body, the catheter was coated with wax and filled with Ringer's solution containing 0.2 per cent liquid.* The exterior end of the catheter was fitted with a rubber tube closed with a clamp; releasing the latter allows blood to be drawn from the vena cava, as it is filled with blood from the renal veins exclusively. In order to avoid dilution of the blood with the aqueous solution present in the catheter, the first 6 cc. of fluid drawn, representing 3 times the volume of the catheter plus rubber tube, were discarded. At the conclusion of each collection, the catheter was flushed with physiological saline solution, after which it was refilled with the Ringer's solution containing liquid.

The urine was collected for periods of time varying from 21 to 50 minutes, care being taken not to introduce inaccuracies due to incomplete emptying of the bladder. The samples of renal venous and arterial (from the femoral artery) blood to be used for the comparative determinations of their apparent creatinine content were drawn as closely as possible to the middle of the periods of collection of the urine samples. The blood was mixed with 2 mg. of dry potassium oxalate per cc. in order to prevent coagulation.

Apparent creatinine was determined in a 1:10 tungstomolybdic acid filtrate prepared from blood the same day the experiment had taken place. It has been the practise to allow at least 1½ hours to elapse after the addition of the sulfuric acid. The precipitated proteins were then centrifuged off and the supernatant fluid filtered through acid-washed Schleicher and Schüll filter paper No. 589²; in this condition the filtrate could be kept in the ice box for several days without apparent deterioration. The object of this combination of centrifugation and filtration was twofold: it provided larger quantities of filtrates than would have

* A synthetic anticoagulant manufactured by Hoffmann-La Roche, Inc.

been obtained by filtration alone and eliminated completely the occasional turbidity which might have led to serious errors when the solutions were compared in the photoelectric photometer.

The color was developed strictly in accordance with the Folin and Wu (1919) technique, the picric acid used having been purified by the sodium carbonate method of Benedict (1929). Simultaneously with the unknowns, one standard lower (equivalent to 1 mg. per cent in the blood) and one higher (equivalent to 2 mg. per cent in the blood) than the expected creatinine content of the blood filtrates were prepared. The filtrates were centrifuged during the latter part of the time allowed for the development of the color. All solutions were read in the photoelectric comparison photometer referred to above, with a Jena glass VG2 as a light filter, in either one of the following orders of sequence:

lower standard		lower standard
venous sample		arterial sample
arterial "		venous "
higher standard	or	higher standard
arterial sample		venous sample
venous "		arterial "
lower standard		lower standard

By averaging the readings thus obtained, one eliminates the disturbing factor which the increase in color on standing otherwise would introduce into these comparative determinations. Before beginning the readings on the creatinine solutions, the cup to be used for the determinations was filled with 0.025 N dichromate solution, set at 15.0 mm., and the other cup containing the same dichromate solution adjusted so as to obtain the initial condition of balance (see Goudsmit and Summerson (1935) p. 428). Then the readings on all creatinine solutions were made. It is obvious that any other solution might have been set at any other convenient depth of layer without affecting the results in the least: the initial setting is mentioned only because in the course of the performance of many hundreds of creatinine determinations on 1:10 blood filtrates, it has been found to be a very practical one.

The apparent creatinine value was calculated as

$$U = A + (B - A) \frac{\frac{1}{r_U} - \frac{1}{r_A}}{\frac{1}{r_B} - \frac{1}{r_A}}$$

where U designates the concentration of the unknown, A and B the concentrations of the lower and the higher standard, respectively, and r_A , r_B , and r_U the readings on the lower standard, the higher standard, and the unknown solution, respectively. As an analysis will readily show, this formula, inasmuch as it eliminates the effect of the color of the alkaline picrate on the proportionality of the readings, might be considered the mathematical equivalent of the colorimeter with the Bürker optical system used by Hayman, Johnston, and Bender (1935), or the Hastings-Duboscq arrangement employed by Van Slyke, Hiller, and Miller (1935). Repeated determinations on solutions with known creatinine content have shown the essential correctness of the formula given. However, a very small deviation (not exceeding 0.002 mg. per cent) consistently present in the average of a sufficiently large number of determinations has been noticed. Since two creatinine values not farther apart than those which form the basis for the present study are practically equally affected by this deviation, it has not been taken into consideration for the calculation of the figures to be presented. No experiments have been undertaken as yet to ascertain whether this departure from the "theory" is caused by the presence of some theoretical factor not taken into consideration or merely reflects a slight irregularity in the measuring apparatus.

The urine was analyzed for creatinine in a portion representing 2 minutes excretion and was compared against two suitable standards, both standards and unknown having been made up to a volume of 100 cc. The comparison was performed with the photometric photometer in a way entirely analogous to the one described for smaller amounts of creatinine, and the calculation from the photometer readings was made with the aid of the same formula.

Urea was determined by the method of Van Slyke and Cullen (1914, 1916).

RESULTS

The results, which have been summarized in Table II, bring out in the first place that, in all thirty instances in which the apparent creatinine in the arterial blood has been compared with that in the renal vein, an appreciable decrease has been observed

TABLE II
Showing Decrease of Apparent Creatinine and Urea in Blood of Renal Vein As Compared with Blood of Artery

Dog, date, and diet	Urine		Apparent creatinine				Creatinine excretion per min.	Urea					Urea excretion per min.	Blood flow through kidney		
	Length of period	Volume	In artery	In renal vein	Absolute difference	Extraction percentage		In artery	In renal vein	Absolute difference	Extraction percentage	Calculated from (11) and (13)		Calculated from (6) and (8)	Theory	
																cc.
(1) Dog S-6, ♀; Mar. 3, 1936; 16.5 kilos. 3 last days before experiment, casein-cracker meal-lard diet	30	0.35	1.38	1.26	0.12	8.50	33	29.1	26.3	2.8	10.3	8.0	285	275	247	
	50	0.23	1.49	1.24	0.25	16.80	305	25.4	23.3	2.1	8.2	6.8	325	120		
	30	0.32	1.43	1.28	0.15	10.50	41	25.1	22.0	3.1	12.3	9.7	310	275		
Dog PE-4, ♀; Mar. 11, 1936; 18.5 kilos. No special diet	25	0.12	1.23	1.12	0.11	9.00	39	24.3	22.7	1.7	7.0	5.7	335	355		
	25	0.12	1.30	1.21	0.09	7.00	34	24.15	22.6	1.55	6.4	5.9	385	375		
	24	0.12	1.30	1.17	0.13	10.00	29	24.7	23.0	1.7	6.7	5.4	330	225		
Dog PE-3, ♀; Mar. 16, 1936; 18.1 kilos. 10 last days on casein-cracker meal-lard diet	25	0.11	1.32	1.12	0.20	15.20	305	24.0	22.2	1.8	7.5	5.3	295	150	277	
	25	0.08	1.32	1.12	0.20	15.20	215	24.4	22.85	1.55	6.0	3.2	205	105		
	25	0.16	1.15	1.05	0.10	8.80	355	23.6	21.9	1.7	7.2	2.0	115	355		
	25	0.20	1.14	0.97	0.17	15.00	295	23.1	20.9	2.2	9.5	4.2	190	175		
	25	0.23	1.13	1.03	0.10	8.90	315	23.4	22.0	1.4	6.0	4.0	290	315		
	25	0.19	1.15	0.99	0.16	14.00	22	23.9	23.2	0.7	3.0	1.8	255	140	271	

Dog D.Ph., ♂; Apr. 3, 1936; 24 kilos. Meat scraps	25	0.16	1.57	1.36	0.21	13.40	745	25.9	24.3	1.7	6.5	13.2	780	355
	25	0.22	1.58	1.44	0.14	8.90	735	25.0	23.1	2.0	8.0	14.4	720	525
	25	0.37	1.49	1.35	0.14	9.30	765	23.9	21.8	2.2	8.3	18.1	825	550
	25	0.43	1.50	1.33	0.17	11.30	765	22.9	20.4	2.6	11.3	16.4	640	450
	25	0.48	1.50	1.37	0.13	8.70	745	21.9	18.9	3.0	13.6	16.2	540	575
Dog PE-6, ♀; Apr. 13, 1936; 18.5 kilos. Mixed diet; fasting last 24 hrs.	25	0.77	1.17	1.04	0.13	11.10	51	20.8	18.8	2.0	9.6	14.1	705	390
	25	1.48	1.16	1.02	0.14	12.10	65	19.1	16.7	2.4	12.6	21.0	875	465
	25	1.59	1.15	1.03	0.12	10.50	62	17.5	14.9	2.6	14.9	17.6	675	520
	25	1.10	1.10	1.02	0.08	7.30	59	17.1	14.7	2.4	14.0	15.7	655	740
	25	0.90	1.07	0.94	0.13	12.10	58	16.8	14.3	2.5	14.9	13.2	530	445
Dog PE-9, ♀; May 4, 1936; 21.5 kilos. Restricted pro- tein; casein-cracker meal- lard diet. Right kidney re- moved 3 wks. prior to ex- periment	28	0.93	1.10	0.99	0.11	10.0	*	22.6	21.3	1.3	5.8	*		
	22	1.16	1.15	1.01	0.14	12.2	*	22.8	20.3	2.5	11.0	*		
	25	1.23	1.15	1.00	0.15	13.00	47	21.4	18.5	2.9	13.5	10.5	360	310
	25	1.48	1.08	0.97	0.11	10.20	42	20.9	18.9	2.0	9.6	10.9	545	380
	35	1.81	1.05	0.97	0.08	7.60	45	19.3	18.4	0.9	4.7	10.4	1155	560
	24	1.54	1.13	0.99	0.14	12.40	47	18.1	16.5	1.6	8.8	10.7	670	340
	21	1.18	1.08	0.98	0.10	9.30	40	17.8	16.0	1.8	10.1	9.4	520	400
	23	1.43	1.03	0.93	0.10	9.70	41	16.9	15.3	1.6	9.5	9.3	580	410

in the latter. This decrease, expressed as percentage of the arterial apparent creatinine level (thus, "extraction percentage" in the sense of Van Slyke, Rhoads, Hiller, and Alving (1934)) has varied from 7.3 to 16.8, with a mean value of 11.0 and a standard deviation of 2.5. It may be recalled here that in the introduction we put the percentage extraction of pure creatinine, a substance evenly divided over plasma and corpuscles, at approximately 11 per cent. It is very interesting to note that apparently the chromogenic material of the blood has the same extraction percentage. Creatinine up to the present time has been the only substance not foreign to the body which, at least in the dog, is known to be excreted by glomerular filtration exclusively and not to be reabsorbed by the tubules. The assumption that the mechanism of excretion of the chromogenic material likewise is one of exclusive glomerular filtration is very tempting in view of the evidence presented.

Another fact brought out by analysis of Table II is that, if the blood flow through the kidney is calculated as the quotient of the amount of creatinine excreted per minute divided by the absolute difference in apparent creatinine content of the femoral artery and the renal vein (as has been done in Column 15), there is in general a fairly satisfactory agreement between this figure and what, in view of the estimation outlined earlier in this paper, it might be expected to be (Column 16). It may be of interest to note that such agreement is better in the experiments on the first three dogs than in those on the latter three in which the volume of urine had been considerably increased by the administration of hypotonic Ringer's solution. In the latter instance, however, the blood flow through the kidney, calculated on the basis of the arterio-venous difference in urea and its excretion per minute, deviates more from the theory (Column 16) than does the blood flow calculated from the data on creatinine.

In the communication of Van Slyke, Hiller, and Miller (1935) referred to before, it is stated that there is no connection between the level of urea, creatinine, inulin, or ferrocyanide in the plasma and the percentage of it removed during one passage through the kidney. We have analyzed our data in order to find out whether the same situation applies to the chromogenic material. From our results we get the impression that a correlation between the

level of apparent creatinine in the blood and the extraction percentage probably does not occur either. Thus the average extraction in seven observations, in which the arterial creatinine varied from 1.40 to 1.59 mg. per cent, amounts to 11.3 per cent, whereas the average of seventeen observations at an arterial level between 1.00 and 1.19 mg. per cent was 10.6.

Additional evidence that the collections of blood were truly representative may be found in the fact that the extraction percentage of urea (mean 9.3, standard deviation 3.1) agrees reasonably well with the one found by Van Slyke, Hiller, and Miller (mean 8.3, standard deviation 2.0).

DISCUSSION

The evidence presented would indicate that in the dog the creatinine appearing in the urine is derived from the chromogenic material in the blood. This finding obviously cannot be interpreted as affording any definite evidence as to whether the chromogenic substance in blood is or is not true creatinine. It is a well known fact that a great many substances more or less related to creatinine will give a color on the addition of alkaline picrate, and thus it is hardly surprising to find that the chromogenic material should be considered as the probable source of urinary creatinine.

The chromogenic material is eliminated from the blood by the kidney of the dog to an extent closely approximating if not equaling the part of any substance excreted in the glomerular filtrate. The excretion by glomerular filtration without reabsorption represents the highest known extent of elimination of a physiologically occurring substance by the kidney of the dog. Thus, the results obtained in the present work would seem to indicate that *practically all* the chromogenic material and not merely a fraction thereof would have to be considered as serving as the source of urinary creatinine.

Shannon, Jolliffe, and Smith (1932) have made an attempt to throw some light on the excretion of endogenous creatinine in a study in which they compared the excretion of creatinine with the level of such fraction of the chromogenic material of the blood as could be recovered from Lloyd's reagent after this had been shaken with blood filtrates. Considering all chromogenic material

released to be creatinine, they come to the conclusion that "there might be enough creatinine in the blood to account for the normal excretion of this substance." However, the interpretation that the chromogenic material released under these circumstances is creatinine does not seem to be justified by the results of Behre and Benedict's and Gaebler's extensive investigations. The fallacy of this type of reasoning was emphasized in Behre and Benedict's more recent discussion (1935). Possibly the statement by Shannon, Jolliffe, and Smith (1932) that they considered their "experimental method none too good, and at best . . . quite non-specific" could be taken as the expression of their willingness to revise their stand on the basis of a reconsideration of the underlying chemical evidence.

It would seem that no investigation limiting itself to a comparison of the level of creatinine-like substance in arterial or non-renal venous blood and the rate of excretion of creatinine would be likely to succeed in suggesting a relationship between these two substances beyond the one displayed by the reaction with alkaline picrate, because under physiological conditions variations occurring in the quantities of either of these substances are not large enough to be correlated with each other with any significant degree of probability. Comparative determinations of the level of apparent creatinine in the blood entering and leaving the kidney, such as form the basis of the evidence presented in this study, can be expected to furnish more information in a problem of this type. It would seem that the results of this investigation have justified such expectations.

SUMMARY

By comparison of the apparent creatinine content of renal venous blood and of arterial blood, it was found that the concentration in the venous blood was consistently lower. The order of magnitude of this difference is such as might be expected if one assumes that the substance responsible for Jaffe's reaction in the blood (apparent creatinine) is the precursor substance of urinary creatinine. The first part of the mechanism of the excretion of the chromogenic material is probably not different from that of ingested creatinine.

These observations do not have a direct bearing on the question of the nature of the chromogenic material in the blood.

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GLYCOGEN DISAPPEARANCE AND CARBOHYDRATE OXIDATION IN HYPOPHYSECTOMIZED RATS*

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Determinations of the respiratory quotient, carried out by Fisher and Pencharz (1), indicated that hypophysectomized rats oxidized a larger proportion of carbohydrate during fasting than did normal rats. At the same time it was established by Russell (2) that hypophysectomized rats, while having normal carbohydrate levels in blood, liver, and muscle when well fed, suffered a progressive fall in these levels to subnormal values during even short fasting periods. These results made it desirable to find out whether the abnormal loss of body carbohydrate during fasting could be accounted for by the increased carbohydrate oxidation, or whether another explanation would have to be sought for the apparent inability of hypophysectomized rats to maintain their carbohydrate stores.

The experiments were so arranged that the amount of carbohydrate which disappeared and which was oxidized could be determined simultaneously on the same animal, a period of fasting being chosen during which practically all of the carbohydrate remaining available for utilization by the rats was in the form of muscle glycogen. Three series of determinations were carried out—on normal rats, on hypophysectomized rats, and on hypophysectomized rats treated with an anterior lobe extract which had been previously observed (3) to prevent the loss of muscle glycogen during fasting.

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EXPERIMENTAL

The animals used were young male rats of the hooded strain, weighing from 140 to 170 gm. when fasted. The hypophysectomies were performed by the parapharyngeal route and the completeness of the operation was checked at autopsy in all cases. The hypophysectomized animals were used from 15 to 25 days after the operation, after maintaining constant weights for several days. The diet previous to these experiments was a ration high in carbohydrate.

The lengths of the preliminary fasting periods were so chosen that at the start of the experimental periods the glycogen contents of muscle in the three series were as nearly as possible alike. On the basis of previous work (2), the following fasting periods were used: normal rats 16 to 18 hours, untreated hypophysectomized rats 9 to 10 hours, and operated rats treated with anterior lobe extract, 12 hours. The blood sugar and liver glycogen were determined at the end of each experiment and found to be at the fasting levels, the average values for the three series being for liver glycogen 100, 15, and 10 mg. per cent, and for blood sugar 108, 66, and 56 mg. per cent for normal, hypophysectomized, and treated hypophysectomized animals, respectively.

In order to determine the glycogen content of muscle tissue in each rat at the start of the experiment, use was made of evipal (sodium N-methylcyclohexenylmethylbarbituric acid), the action of which is of very short duration. With the intraperitoneal administration of this substance in amounts of 9 mg. per 100 gm. to normal or 6 mg. per 100 gm. to hypophysectomized rats, a prompt anesthesia was produced, which was deep enough to allow the excision of a gastrocnemius muscle without twitching. After removal of the muscle for analysis, the small amount of bleeding was easily stopped with cotton wads, the incision sewed up, and the animal put in a respiratory chamber, where it recovered from the anesthesia in a short time, before the determination of the respiratory metabolism was started.

After the measurement of respiratory metabolism, the rat was anesthetized again with evipal or nembutal, and with the usual precaution the remaining gastrocnemius was removed, blood obtained from the vena cava for sugar, and the liver obtained for glycogen analysis. The glycogen determinations were made by

the cold KOH method, the glucose being determined with the 1 gm. KI reagent of Shaffer and Somogyi (4).

The respiratory metabolism was determined by the open circuit gravimetric method over periods of 6 to 7 hours. The animal chambers were fitted with wire screen bottoms so that urine excreted during the experimental periods was obtained for total nitrogen analyses, made by the Kjeldahl method. The bladders of the rats were emptied as completely as possible before the start of the periods and again at the end, but even so the variations in urinary nitrogen obtained in the short period of the tests were large; so that for the calculations of the non-protein respiratory quotients the average value of 3.3 mg. of N per 100 gm. per hour was used. In the operated animals treated with the anterior pituitary extract, the preparation used so suppressed the urine volume that the nitrogen values obtained, in the neighborhood of 1 mg. per 100 gm. per hour or less, were not considered valid.

The anterior lobe preparation used was a neutralized alkaline extract of beef pituitary.¹ It was administered intraperitoneally in doses of 0.5 cc. each to each rat at 18 hours and 1 hour before the start of the experiments; it contained about 2 per cent organic material, so that the total dosage was about 20 mg. of anterior lobe substance.

The observations made in individual experiments of the change in muscle glycogen content and of the oxygen consumption and respiratory quotients over the same periods of time are recorded in Table I. It may be seen upon inspection that there is a good correlation between the loss of muscle glycogen and the respiratory quotient; the correlation coefficient between the figures in Columns 3 and 6 of the three series is actually $+0.82$.

The averages of the three experimental series are shown in Table II, including calculations of the amount of carbohydrate which disappeared (Column 4) and which was oxidized (Column 9). The figures for actual amounts of carbohydrate which disappeared are necessarily only approximate, being based on the assumption that the observed glycogen content is distributed in 50 per cent of the body weight, but the error should be about the same in all series,

¹ Details concerning the action of the extract on the carbohydrate levels of hypophysectomized rats are given in the paper of Russell and Bennett (3).

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rendering comparisons possible. In the first two series of experiments carbohydrate oxidation exceeded the amount of glycogen

TABLE I

Loss of Muscle Glycogen and Respiratory Quotients of Hypophysectomized and Normal Rats during 6 to 7 Hours of Fasting

	Muscle glycogen				O ₂ consumption per 100 gm. per hr. (5)	R.Q. (6)	Urine N per 100 gm. per hr. (7)
	Initial	Final	Difference	Lost per 100 gm. per hr. (4)			
	(1)	(2)	(3)				
	mg. per cent	mg. per cent	mg. per cent	mg.	cc.		mg.
Normal rats, fasted 16 to 18 hrs. at start		485			160	0.738	4.8
	534	492	42	3.9	155	0.741	3.2
	575	525	50	4.5	146	0.730	2.7
	575	530	45	4.2	138	0.732	3.6
	568	542	26	2.0	157	0.702	3.6
	492	463	29	2.2	148	0.734	2.6
	612	562	50	4.3	146	0.751	3.8
	476	475	1	0.0	116	0.733	2.0
	632	622	10	0.8	137	0.727	2.8
Hypophysectomized rats, fasted 10 hrs. at start	556	347	209	16.4	100	0.785	2.1
	504	355	149	11.1	99	0.741	2.9
	408	330	78	6.8	113	0.750	3.8
	514	297	217	16.7	107	0.785	5.0
		318			115	0.760	3.8
	366	188	178	12.3	99	0.755	3.6
	347	217	130	10.0	87	0.785	4.4
Hypophysectomized rats, treated with anterior lobe extract, fasted 12 hrs. at start	465	367	98	7.3	75	0.731	*
	501	484	17	1.2	93	0.721	
	491	405	86	5.9	103	0.712	
	500	392	108	7.4	91	0.743	
	445	484	(38)	0.0	80	0.716	
	502	471	31	2.5	92	0.714	
	486	381	105	6.4	97	0.722	
	527	463	64	5.0	80	0.747	

* Owing to the suppressing action of the extract on urine volume, values obtained on urine N in this series are not considered valid.

which disappeared. In the case of the treated hypophysectomized rats the non-protein quotient and the amount of carbohy-

drate oxidized were not calculated, because accurate figures for urinary nitrogen could not be obtained.

The discussion which follows is based on the findings recorded in Table II that hypophysectomized rats, during a short fasting period, lose much more carbohydrate and have higher respiratory quotients than normal rats and that substitution therapy with an anterior lobe preparation retards very markedly the disappearance of carbohydrate and simultaneously depresses the respiratory quotient to the normal fasting level.

TABLE II

Carbohydrate Disappearance and Oxidation during Fasting

Based on averages of figures in Table I.

	Muscle glycogen				O ₂ consumption per 100 gm. per hr.	R. Q.	Urine N per 100 gm. per hr.	Non-protein R. Q.	Carbohydrate oxidized per 100 gm. per hr.
	Initial (1)	Final (2)	Difference (3)	Lost per 100 gm. per hr. (4)					
	mg. per cent	mg. per cent	mg. per cent	mg.	cc.		mg.		mg.
Normal rats.....	558	522	36	2.7	145	0.731	3.2*	0.720	7.5
Hypophysectomized rats	450	293	157	12.2	103	0.767	3.4*	0.759	19.9
Hypophysectomized rats with anterior lobe extract	491	431	60	4.5	89	0.726			

* The average value of 3.3 mg. of N per 100 gm. per hour was used in calculating non-protein R. Q.

DISCUSSION

It has been considered by several workers that the frequent spontaneous hypoglycemiae and occasional low glycogen reserves found in hypophysectomized animals are due to a deficient new formation of glucose from non-carbohydrate sources. Evidence has also been presented that when gluconeogenesis is stimulated by phlorhizin injections, hypophysectomized animals fail to show the usual rise in nitrogen metabolism (5). Finally, the ameliora-

tion of pancreatic diabetes following removal of the hypophysis has been attributed to decreased gluconeogenesis.

The present experiments indicate that in hypophysectomized rats, under fasting conditions, factors other than gluconeogenesis play a rôle. There was no significant decrease in nitrogen metabolism such as one would expect to be associated with deficient gluconeogenesis. The rise in respiratory quotient coincident with the increased rate of disappearance of carbohydrate reserves in the operated animals and—under treatment with anterior lobe extract—the prompt regression of the quotient to low fasting values occurring simultaneously with the preservation of glycogen levels are facts which point to some change in carbohydrate oxidation.

It has been shown previously (6) that there exists in normal rats a mechanism which leads to the preservation of glycogen during fasting. During the first few hours of fasting a considerable amount of carbohydrate undergoes oxidation, about 23 per cent being derived from liver glycogen and 77 per cent from the rest of the body. Soon, however, an adjustment takes place. Carbohydrate oxidation diminishes progressively and the decrease in the glycogen stores is slowed down correspondingly until after 18 to 24 hours of fasting a stationary state is reached, when the new formation of carbohydrate (from protein and glycerol) balances the small amounts of carbohydrate which the animals continue to oxidize. Comparison of the glycogen content of normal rats after 24 and 48 hours of fasting shows only a small loss of glycogen (an average of 22 mg. per 100 gm. of rat) and such animals have respiratory quotients at the fat-protein level.

It now appears that the pituitary is concerned in the regulatory mechanism which leads to the preservation of glycogen during fasting. In hypophysectomized rats the glycogen of liver and muscle disappears at a greater rate and the respiratory quotient remains at a higher level than is the case in normal rats, suggesting that the pituitary exerts a restraining influence on carbohydrate oxidation and thereby enables the animal to maintain its glycogen stores during fasting. This supposition is strengthened by the fact that substitution therapy with an anterior lobe extract depresses the respiratory quotient to the normal fasting level and

at the same time retards very markedly the disappearance of glycogen. An alternate explanation would be that the anterior lobe extract retards the mobilization of glycogen, which would secondarily influence carbohydrate oxidation. Preliminary experiments indicate, however, that when normal rats are treated with anterior lobe extract and are then given a glucose meal, they show a marked depression in carbohydrate oxidation.

No studies have been carried out regarding the effects of various periods of fasting on the carbohydrate levels and respiratory exchange of hypophysectomized animals other than the rat; it is possible that in conditions in which spontaneous hypoglycemia is known to occur after hypophysectomy in other species, results similar to those in the rat would be found. It should be considered, however, that, when large laboratory animals such as dogs, cats, or rabbits are fasted the usual lengths of time, they are not comparable in their nutritional state to a much smaller animal such as the rat. It is possible that some other phenomena relating the anterior pituitary to carbohydrate metabolism may find their explanation in an effect on carbohydrate oxidation.

SUMMARY

1. The disappearance of muscle glycogen, the oxidation of carbohydrate, and the excretion of urinary nitrogen were determined on rats which were normal, hypophysectomized, or hypophysectomized and then treated with an anterior lobe extract.

2. With about the same amounts of glycogen available at the start of the fasting period, the hypophysectomized rats lost much more muscle glycogen and had correspondingly higher respiratory quotients than the normal animals.

3. Treatment of the hypophysectomized rats with an alkaline extract of beef anterior lobes diminished the loss of muscle glycogen and restored the respiratory quotients to the levels observed in normal rats.

4. The hypophysectomized rats excreted the same amount of nitrogen during the experimental period as the normal animals.

5. It is concluded that hypophysectomized rats suffer a defect in the mechanism by which normally carbohydrate oxidation is depressed and carbohydrate levels thereby maintained during fast-

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ing, and that anterior lobe extracts are able to restore this function, apparently by exerting a depressing effect on carbohydrate oxidation.

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DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

VII. STUDIES IN BILE ACID FORMATION*

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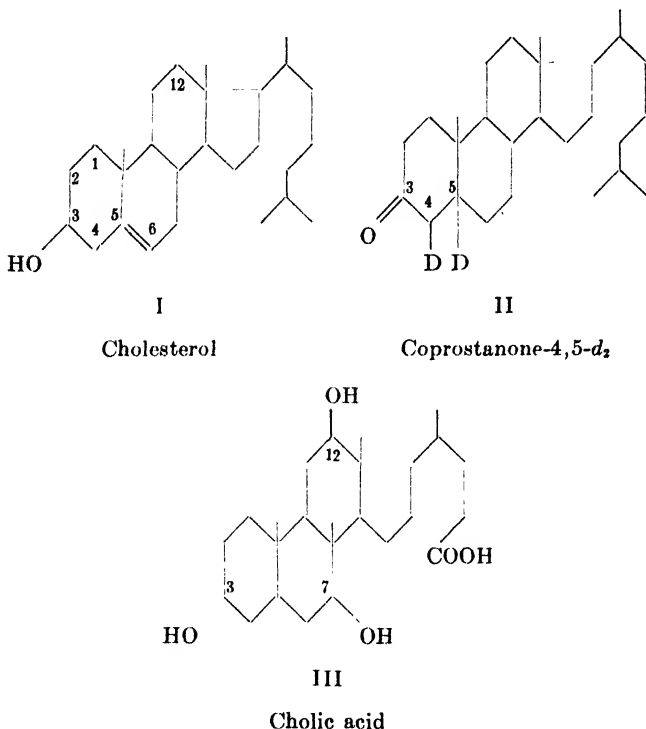
It has been frequently suggested that cholesterol is the common precursor of all the steroids¹ present in the animal organism. In a previous communication (1) it has been shown, by the use of deuterium as an indicator, that the ketones, cholestenone and coprostanone, could be converted to coprosterol by the organism. The hypothesis was there discussed that these ketones, in addition to being intermediates in the formation of coprosterol, might also be concerned in the transformation of cholesterol into other steroids. The conversion of cholesterol to bile acids would involve the hydrogenation of the double bond to give an analogous *cis*-decalin configuration, as in the case of coprosterol.

To test the possibility of a conversion of coprostanone into the bile acids a series of dogs with bile fistulas were injected with a suspension of the same coprostanone-4,5-*d*₂ used in our coprosterol experiments (1). The bile of these animals, after injection, contained an unsaponifiable substance (probably unaltered coprostanone) which contained deuterium. This substance could be separated from the bile acids only with considerable difficulty, and in our first experiment, in which the bile acids were not rigorously purified, the presence of a small amount of deuterium in the

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¹ The term "steroid" has been proposed by R. K. Callow (private communication) as "a generic name for the group of compounds comprising the sterols, bile acids, heart poisons, saponins and sex hormones" all of which contain the reduced cyclopentenophenanthrene ring system.

cholic acid fraction suggested that a partial conversion of coprostanone to cholic acid had occurred. In subsequent experiments, however, analytically pure cholic acid was obtained by recrystallization, and these samples contained no detectable amounts of deuterium. This result again illustrates the necessity of working with only highly purified compounds in those experiments in which deuterium is used to trace biological conversions. The



presence of the deuterium-containing substance in the fistula bile of our dogs is proof that the injected coprostanone (or a substance derived from it) had passed through the liver without being utilized by this organ in the formation of cholic acid.

In experiments in which biological transformations are traced by the conversion of deuterium-containing substances into other compounds, it is necessary to exclude the possibility of chemical reactions occurring at those carbon atoms to which the deuterium

is attached, since such reactions might lead to the elimination of deuterium from the molecule (2). In the present case the loss of both deuterium atoms from the coprostanone-4,5- d_2 molecule seems highly improbable. While the deuterium atom at carbon atom 4 may exchange with the hydrogen of the solvent by enolization of the carbonyl group at position 3 (1), no reaction in the hypothetical conversion of coprostanone to cholic acid (shortening of the side chain, reduction of the carbonyl to a hydroxyl group, and introduction of two hydroxyl groups at carbon atoms 7 and 12) would involve the loss of the stable deuterium atom at carbon atom 5.

The absence of deuterium in the cholic acid isolated in our experiments indicates that coprostanone cannot be converted to cholic acid by the animal organism, and therefore cannot be an intermediate in the hypothetical conversion of cholesterol to cholic acid. These negative results raise the question as to whether the frequently discussed metabolic relationship between cholesterol and the bile acids exists, or whether both groups of substances (and this may also be true of the sex hormones) are not synthesized independently in the animal organism. Further experiments are being carried out in this direction.

EXPERIMENTAL

The bile fistulas were prepared by the method of Rous and McMaster (3), the first injection of coprostanone being given 1 week after the operation. Bile was collected in rubber balloons which were emptied daily under aseptic conditions. The dogs were fed the usual diet of raw meat with the addition of a small quantity of cod liver oil. Only those animals were used in which the bile was clear and showed no signs of infection. One dog was discarded because of a progressive decrease in the daily excretion of bile.

The coprostanone-4,5- d_2 was prepared by the method of Schoenheimer, Rittenberg, and Graff (1). It contained 3.44 atoms per cent of deuterium. The emulsion was obtained by dissolving 2 gm. of coprostanone-4,5- d_2 and 2 gm. of purified lecithin in ether. The ether was removed, the residue stirred up with 100 cc. of warm water (65°), and then passed through a mechanical emulsifier at 65°. The resulting creamy emulsion, which appeared homo-

geneous under the microscope, was injected immediately after its preparation.

Injections were made into the jugular vein, without anesthesia, the administration of 30 cc. of the emulsion requiring somewhat longer than 30 minutes. The dogs showed no unfavorable response and in no case was there a decrease in the bile flow after injection. The injections were made on alternate days, the bile being collected until 24 hours after the final injection.

The experimental data are summarized in Table I.

Isolation of Cholic Acid—The bile was added to twice its volume of alcohol, and the solution was brought to a boil and then filtered. The filtrate was brought to dryness; the residue was dissolved in 20 per cent NaOH and hydrolyzed for 24 hours on the steam bath. The solution was acidified with HCl; the gummy

TABLE I
Bile Acids Isolated after Coprostanone Injection

Experiment No.	Weight of dog	No. of injections given	Total amount of coprostanone injected	Volume of bile used for isolation	Amount of bile acid isolated
	kg.		gm.	cc.	gm.
1	11	2	1.5	130	0.70
2	9	2	1.1	160	1.4
3	11	4	4.0	670	3.8

precipitate was centrifuged down, washed several times with water, and dissolved in dilute aqueous ammonia. The fatty acids and a small amount of desoxycholic acid were precipitated with $\text{Ba}(\text{OH})_2$. The mother liquor was acidified with H_2SO_4 and extracted with ether. The ether was washed free from acid with water and dried over Na_2SO_4 . The material present in this ether solution consisted principally of cholic acid.

The ethereal solution obtained in this manner from the bile of Dog 1 was brought to dryness and the residue extracted for 1 hour with boiling xylene in the expectation that this procedure would remove the remaining fatty acids and unsaponifiable material (4). The 0.700 gm. of yellow crystalline material remaining after the extraction melted indistinctly at 180–190°. It contained 0.06 atom per cent of deuterium.

The bile of Dogs 2 and 3 was treated similarly, the cholic acid, however, being isolated in analytically pure form after the unsaponifiable fraction of the bile was removed first by ether extraction. This was done by continuous ether extraction of the alkaline hydrolysate of the bile for 24 hours. The residue from the ether was again saponified with 7 per cent KOH in methyl alcohol. The unsaponifiable material thus obtained (0.262 gm.) consisted of a yellow semicrystalline mass. It contained 1.1 atoms per cent of deuterium.

The alkaline hydrolysate of the bile remaining after ether extraction was acidified with HCl. The precipitate was centrifuged down, washed with water, and dissolved in dilute ammonia. The solution was acidified with H_2SO_4 and extracted with ether. The ether was washed free of acid and dried over Na_2SO_4 . To obtain pure cholic acid this ether extract was brought to a small volume and placed in the refrigerator for several days. The supernatant ether was then decanted from the heavy crystalline prisms of cholic acid adhering to the walls of the flask. The crystalline material was washed with ice-cold ether and recrystallized three times from aqueous alcohol. After drying *in vacuo* over P_2O_5 for 8 hours, both samples (Dogs 2 and 3) had a melting point of 196° . The deuterium content of both samples was 0.00 ± 0.03 atom per cent.

Analysis of Cholic Acid from Dog 3—Calculated. C 70.54, H 9.87
Found. " 70.35, " 9.78

SUMMARY

Dogs with bile fistulas were injected intravenously with an emulsion of coprostanone-4,5- d_2 . A deuterium-containing unsaponifiable substance (probably unaltered coprostanone) was subsequently found in the fistula bile. The cholic acid isolated from the bile in analytically pure form did not, however, contain any deuterium. The experiments indicate that coprostanone is not an intermediate in the hypothetical conversion of cholesterol into cholic acid.

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COMPARISON OF GLYCINE AND GUANIDOACETIC ACID AS PRECURSORS OF CREATINE*

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Of the many alleged precursors of creatine only two compounds, namely glycine and guanidoacetic acid, have been found to increase the urinary creatine output with any degree of regularity (1). However, as previously stated, this does not necessarily prove their conversion into creatine; at best the evidence is only presumptive of extra creatine production. Indeed, in relation to the amount fed, the increment in the case of glycine is so small as to suggest that it may be due, not to its conversion into creatine, but perhaps to other causes, such as stimulation of cellular metabolism, increased renal function, or a "washing out" of free creatine from the tissues.

The evidence that a given compound is a precursor of creatine would be materially strengthened by the demonstration that it not only increases the urinary output, but at the same time definitely raises its content in the tissues. According to Beard and associates (2, 3) the administration to young rats of 1 gm. of glycine resulted in an average increase of 15.4 per cent in muscle creatine and an augmented urinary output, averaging 35.9 per cent. Feeding 1 gm. of guanidoacetic acid is said to have raised the level of muscle creatine by an average of 48.5 per cent. The force of these observations seems to be weakened, however, by the fact that Beard and coworkers obtained equally astounding changes on

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feeding various proteins, amino acids, and other compounds, some of which are chemically quite unrelated to creatine and creatinine.

The data in the earlier literature (4) purporting to show that guanidoacetic acid produces a significant increase in creatine excretion is fairly convincing, but as regards the effect on muscle, the results have often been negative and far from uniform. Nevertheless, considering the available data as a whole, the evidence for a direct conversion into creatine seems to be somewhat more definite for guanidoacetic acid than for any other compound studied from this standpoint. The current interest in glycine as a therapeutic agent in muscle disease and the assumption by certain investigators that this amino acid is a precursor of creatine are among the reasons which stimulated the present attempt to clarify certain questions by comparing the effects of feeding glycine, guanidoacetic acid, and creatine on the distribution of the latter in the tissues. As the work progressed, it was realized that the problem might be further elucidated by determining in addition the guanidoacetic acid content of the tissues.

Methods for Preliminary Experiments

For each series of observations twelve rats of the same sex and approximately the same age and weight were selected. On the evening preceding the experiment a specimen of muscle was excised from the right thigh of each rat, after which the operative wound was carefully sutured. There was a minimum of trauma connected with this procedure, the animals appearing quite normal within a half hour after the operation. This was performed under light ether anesthesia. On the following morning eight of the rats received the test substance, dissolved or suspended in 4 to 5 cc. of water, by stomach tube. The remaining four rats served as controls and received 4 to 5 cc. of water (in later experiments 1 gm. of glucose dissolved in water was given to the control animals). Only one compound was studied at a time, the doses being 50 and 100 mg. for creatine, 100 and occasionally 200 mg. for guanidoacetic acid, and 1 gm. for glycine. It is appreciated that the last dose may be considered very large and unphysiological, a comparable dose in the average man being 300 to 400 gm. However, there was no alternative to the use of an effective dose, amounts much less than 1 gm. having been found not to augment

creatine excretion. As a rule no untoward effects resulted from the glycine; occasionally mild diarrhea developed.

Following passage of the stomach tube a rat was sacrificed at hourly intervals for the first 6 hours, then at 12 and 24 hours. The controls were usually sacrificed at 1, 6, 12, and 24 hours. Muscle for analysis was removed from the unoperated hind limb. The heart, liver, and kidneys were likewise excised for analysis. For the determination of creatine the method of Rose, Helmer, and Chanutin was used (5).

Five series of experiments were performed with glycine, four with guanidoacetic acid, and three with creatine.

Results of Preliminary Experiments

In order to economize space only a brief summary of the results will be given.

Heart—The creatine concentration of the myocardium was not appreciably affected in any of the experiments.

Muscle—The creatine concentration of the muscle was only slightly increased by the administration of creatine, the peak occurring usually between the 3rd and 6th hours (usually about 15 to 20 mg. per 100 gm. in rats receiving 100 mg. of creatine). The effect was evidently transitory, as the concentrations at 12 to 24 hours closely approximated the biopsy specimens. In about half the rats fed guanidoacetic acid the concentration of creatine increased slightly above the biopsy specimen level; in the others the change was even less definite. Most of the data on the glycine-fed rats, as well as in the control animals, differed from the results obtained on the biopsy specimens to the extent to be expected owing to the limitations of the analytical procedure. In some cases the concentration seemed to be definitely below the initial level.

Liver—Following its administration the concentration of creatine in the liver was invariably increased, often attaining values 400 to 500 per cent above normal at 1 to 2 hours. The high level was usually maintained for 4 to 5 hours, after which the concentration rapidly declined, essentially normal values being obtained at 12 to 24 hours. It was surprising to discover, on the other hand, that guanidoacetic acid produced little, if any, change. There was an apparent increase of 3 to 4 mg. per 100 gm. of liver, but

as guanidoacetic acid itself exerts a chromogenic effect with picrate (100 mg. is equivalent to about 4 mg. of creatinine), it is doubtful whether the small increment actually represented creatine. In the glycine-fed animals the creatine concentration either remained unchanged or was slightly lower than in the controls.

Kidney—The creatine concentration of the kidney was significantly elevated in all experiments, the greatest effect occurring, as was to be expected, in the creatine-fed group. In the rats sacrificed at 1, 2, and 3 hours, there was often a 300 to 400 per cent increase (from a normal of 40 to 50 mg. to as much as 180 mg. per 100 gm.). The concentration then diminished at a variable rate, returning to normal within 24 hours and occasionally sooner (12 hours). The administration of guanidoacetic acid likewise produced a sharp rise in kidney "creatine," the maximum increment approximating 80 to 100 per cent and occurring in 6 to 12 hours. By the end of 24 hours the concentration diminished to normal. In the glycine-fed rats the increase in concentration, though definite in about two-thirds of the cases, rarely exceeded the basal level by as much as 50 per cent. The high point was usually attained in 4 to 6 hours.

Although it was considered improbable that more than a fraction of the creatine increment could be due to other substances giving the Jaffe reaction, nevertheless it seemed desirable to determine the amount of guanidoacetic acid accumulating in the kidneys. Weber's procedure (6) was adapted to tissue analysis and proved so satisfactory that its use was extended to include muscle, heart, liver, and alimentary tract.

Method

Approximately 3 gm. of tissue in 50 cc. of 0.4 N HCl are autoclaved at 15 pounds pressure for not more than 5 minutes. This aids in the disintegration of the tissue. After the solution is diluted to 200 cc. with water, the procedure is essentially that employed by Weber for urine. 10 gm. of Lloyd's reagent are added and the mixture stirred continuously for 3 minutes, then filtered on a Buchner funnel, and the residue rinsed with water, slightly acidified with sulfuric acid. The Lloyd's reagent is then suspended in 100 to 150 cc. of water, treated with 10 gm. of barium hydroxide, stirred for 3 minutes, and filtered with suction, the

residue being rinsed several times with water. The barium in the filtrate is precipitated with 40 per cent sulfuric acid, added to the Congo red end-point. After the mixture is diluted to 250 cc. and stirred, 50 cc. are removed and centrifuged. The clear supernatant fluid is separated and made alkaline with basic lead carbonate, after which it is filtered. 25 cc. of the filtrate are shaken with 3 gm. of permutit for 5 minutes and filtered. To 5 cc. of the filtrate (or diluted filtrate if the concentration of guanidoacetic acid proves to be too high) in a large test-tube (200 × 25 mm.) are added 1 cc. of 10 per cent sodium hydroxide and 1 cc. of 0.04 per cent α -naphthol. In our laboratory the tubes are placed in an ice water bath and kept in the refrigerator for at least 15 minutes. To the contents are then added 0.3 cc. of a cooled solution of sodium hypobromite (2 gm. of bromine added to 100 cc. of 5 per cent sodium hydroxide), followed by 1 cc. of 40 per cent urea solution and 10 cc. of water, the last two being likewise chilled. The color which develops in the presence of guanidoacetic acid is compared in the colorimeter with that produced in a standard solution (5 cc. \approx 0.025 mg.) treated in precisely the same manner, except that only 0.2 cc. of sodium hypobromite is used, as suggested by Weber.¹

¹ Weber's method has a high degree of specificity. Such compounds as arginine and methylguanidine which give a direct Sakaguchi reaction are almost completely removed before the color-producing stage is reached. In our hands 100 mg. of arginine, carried through the usual procedure, yielded a color equivalent to not more than 3 mg. of guanidoacetic acid. For all practical purposes there was no interference from the following substances: sarcosine, choline, betaine, creatine, creatinine, glycine, alanine, histidine, guanidine (hydrochloride, carbonate, and acetate), methylguanidine. We have not had an opportunity to investigate the homologues of guanidoacetic acid. The effect of feeding these substances on the composition of the urine was determined in detail. Glycine (two doses of 1 gm.) produced an average increase of 1.2 mg. of urinary guanidoacetic acid per 48 hours. Arginine given in 100 mg. daily doses produced an apparent increase of 0.9 mg. per 48 hours. 50 mg. of guanidoacetic acid given daily for 2 days increased the urinary output by 29 to 33 mg. When 100 mg. were given in a single dose, the excretion was 40 to 50 mg. According to Weber (personal communication) the recoveries of added guanidoacetic acid have been 85 ± 5 per cent. In our hands the recoveries have been more nearly 75 ± 5 per cent. In the data recorded in this paper *no correction* has been made for the probable error.

TABLE I
Changes Produced by Guanidoacetic Acid, Creatine, and Glycine

Rat No.	Average weight	Substance fed	Sacrificed after	Guanidoacetic acid recovered in all-meaty tract	Guanidoacetic acid (G) and creatine (Ci), per 100 gm. tissue								Guanidoacetic acid or creatine recovered in urine	
					Liver			Muscle		Heart	Kidney		G	Ci
					G	Ci	G	Ci (average)	G		Ci			
	gm.		hrs.	mg.	mg.	mg.		mg.			mg.	mg.	mg.	mg.
643 ♀	235	Guanidoacetic acid, 100 mg.	3	13.9	34.9	21.5	+	462	+	43.6	31.3			
644 ♀				25.0	40.7	23.5	+							
645 ♀				26.6	24.0	20.2	++							
646 ♀	237	Same	6	14.7	39.7	25.5	+	494	+	31.2	44.0			
647 ♀				24.5	28.9	20.7	+							
648 ♀				17.5	17.8	23.7	+							
649 ♀	215	Control (water)	6	+	0	15.8	0	502	0	0	26.7			
650 ♀				+	0	14.1	0							
651 ♀				0	0	18.2	0							
652 ♂	373	Guanidoacetic acid, 100 mg.	3	30.5	27.5	27.5	0	487	+	65.9	54.7			
653 ♂				20.3	33.9	36.3	+							
654 ♂				16.8	27.6	27.8	+							
655 ♂	358	Same	6	17.6	23.1	27.7	++	466	+	41.0	60.0	27.8	13.5	11.9
656 ♂				31.5	21.2	15.0	++							
657 ♂				21.8	22.3	24.1	++							
658 ♂	353	Control (water)	6	+	0	21.1	0	456	0	+	43.1			
659 ♂				0	0	20.7	0							
660 ♂				+	0	19.5	0							
664 ♂	363	Guanidoacetic acid, 100 mg.	12	8.5	0	20.8	0	492	+	++	71.0	50.7	28.9	29.9
665 ♂				6.7	0	17.5	0							
666 ♂				++	0	16.7	0							
667 ♂	377	Same	24	1.3	0	14.3	0	488	0	+	41	41.7	50.0	41.7
668 ♂				1.4	0	14.8	0							
669 ♂				5.0	0	15.7	0							
670 ♂	238	Creatine, 100 mg.	3	+	0	56.6	0	482	0	+	91.5		3.5	10.3
671 ♂				+	0	31.6	0							
672 ♂				+	0	49.6	0							

* + denotes a trace and ++ a strong trace. In the analysis of urine, a daily output of guanidoacetic acid of as little as 0.2 to 0.3 mg. may be estimated quantitatively. However, in the analysis of the tissues where about 3 gm. only were available, a trace (+) probably represented 1 to 2 mg. per 100 gm. and a strong trace (++) as much as 5 mg. per cent.

† The creatine recovered from the alimentary tracts of Rats 670, 671, and 672 amounted to 28.1, 23.5, and 23.5 mg., respectively. In Rats 673, 674, and 675 the recoveries were 18.3, 17.7, and 17.1, respectively.

TABLE I—*Concluded*

Rat No.	Average weight	Substance fed	Sacrificed after	Guanidoacetic acid recovered in allimentary tract	Guanidoacetic acid (G) and creatine (Ci), per 100 gm. tissue								Guaninoacetic acid or creatine recovered in urine	
					Liver		Muscle		Heart	Kidney		G	Ci	
					G	Ci	G	Ci (average)		G	Ci			
	gm.		hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
673 ♂	241	Same	6	+	0	24.5	0	474	0	+	73.7		19.8	
674 ♂				+	0	22.0	0							
675 ♂				+	0	27.3	0							
676 ♂				+	0	13.8	0							
677 ♂	258	Glycine, 1 gm.	3	+	0	17.1	0	451	0	+	48.4			
678 ♂				+	0	25.0	0							
679 ♂				+	0	15.3	0							
680 ♂	266	Same	6	0	0	15.1	0	475	0	+	40.3			
681 ♂				+	0	20.0	0							
682 ♂	266	Same	12	+	0	18.8	0	443	0	+	49.7		1.2	
683 ♂				+	0	16.5	0						0.9	
684 ♂				+	0	21.6	0						1.2	

Procedure for Later Experiments

A total of thirty-nine rats was used, these being divided in groups of three. Of the thirteen groups, two were controls, six received guanidoacetic acid (100 mg.), two creatine (100 mg.), and three glycine (1 gm.). The animals were autopsied at 3, 6, 12, and 24 hours, as shown in Table I. Biopsy specimens were not taken in these experiments. For analysis it was necessary to combine the three hearts of each group. The three right kidneys of each group were analyzed together for creatine, the three left for guanidoacetic acid. There were sufficient liver and muscle for separate analyses on each rat. The entire alimentary tract and its contents were removed from each animal and analyzed separately. A number of rats were kept in individual metabolism cages in order to secure their urine for analysis.

Results

Guanidoacetic acid apparently does not occur normally in muscle, liver, and heart in sufficient amounts to be demonstrable by the method used, but in the kidneys it is usually found in

traces, which is not surprising in view of the excretion of about 0.5 mg. daily. Appreciable amounts have also been found in rat testes and intestines. The significance of these findings is under investigation.

It will be noted that the greater part of the guanidoacetic acid disappeared from the alimentary tract during the first 3 hours, after which absorption was more gradual. However, little remained at the end of 24 hours. A considerable amount appeared in the liver, but it is interesting to observe that none was found at 12 and 24 hours. In agreement with the results previously described there was no clear cut rise of the creatine content of the liver, except perhaps in Rats 652 to 654. Guanidoacetic acid likewise appeared in muscle and heart, but in insufficient amounts for quantitative estimation. Considerable amounts occurred in the kidney during the first 6 hours. Its rapid elimination is indicated by the presence of slightly more than traces at 12 and 24 hours. The urine excreted during this time contained as much as 50 per cent of the administered guanidoacetic acid. As in previous experiments the increase in creatine in the kidney (and urine) was much more than could be accounted for on the basis of the chromogenic effect of the acid.

In the creatine-fed rats the results were similar to those previously described (note particularly the changes in the kidney and liver). The liver, muscle, and heart were free from guanidoacetic acid. As in the control rats, a trace of this compound was present in the kidneys. No special significance can be attached to the creatine values obtained for the muscle.

Guanidoacetic acid was likewise absent from the liver, muscle, and heart of the glycine-fed rats. In connection with other work it has been observed that the administration of 1 gm. of glycine increases somewhat the output of guanidoacetic acid. However, the increment, which rarely exceeds 0.5 mg., is probably attributable to the same fundamental causes which increase the creatine excretion. Analyses of various preparations of glycine have yielded negative results for guanidoacetic acid (1).

DISCUSSION

Brand and Harris have stated (7): "Our experiments indicate that the guanidine group of creatine is synthetic in origin and that

glycine is involved in creatine formation." This view is based partly on the discovery of increased creatine excretion as the result of feeding glycine in cases of progressive pseudohypertrophic muscular dystrophy (8) and partly on the observation that the administration of benzoic acid produced a marked drop in creatine excretion, presumably by removing glycine from the metabolic mixture (7, 8). Freiberg and West (9) have shown, however, that in pseudohypertrophic muscular dystrophy the capacity of the organism to produce glycine in response to benzoic acid differs in no way from that in the normal individual. In the rat the administration of benzoic acid has been found not to diminish the output of creatine (unpublished data). Additional evidence of the conversion of glycine is the change in muscle creatine reported by Beard and associates, as previously mentioned.

For the sake of argument suppose it were conceded that glycine is a precursor of creatine and, to be conservative, suppose it were further assumed that the conversion affected only a small fraction, perhaps 5 to 10 per cent of the glycine fed. What changes might then be expected under these circumstances? Muscle comes first to mind because it is so definitely linked with creatine metabolism. Obviously a consistent increase in the creatine content of muscle would point to glycine as a precursor, but to expect that this must necessarily occur would be to ignore certain fundamental principles. The idea that the fate of creatine is restricted to its storage, conversion into creatinine, and excretion has been definitely disproved by Benedict and Osterberg (10), who showed that about two-thirds of the creatine fed to dogs followed other paths in metabolism. The destruction of creatine by the organism has also been observed by Chanutin and Silvette (11) in experiments with nephrectomized rats. These workers had previously determined the conditions under which creatine accumulates in the tissues of the normal rat (12).

In view of the difficulty of demonstrating a definite increase in muscle even when creatine itself is fed in small amounts, a negative result with glycine cannot therefore be considered as crucial evidence against the view that it is a precursor of creatine. It is, however, significant that in the work presented here and elsewhere (13) the results with glycine have been invariably negative, differ-

ing thus from some positive results obtained under similar conditions with creatine and guanidoacetic acid. It is perhaps not unreasonable to suppose that if glycine were converted into creatine and this involved the formation of guanidoacetic acid as an intermediate, the latter would have accumulated in muscle and liver in sufficient amounts to have been detected.

From 40 to 50 per cent of the guanidoacetic acid fed was excreted unchanged. The extra creatine production accounted for an additional 10 to 15 per cent. From the data it would appear that methylation occurred predominantly in the kidney. There is, however, the possibility that at least some of the creatine may have originated in muscle. The concentration of guanidoacetic acid in muscle was probably never in excess of 10 mg. per cent in our experiments. Even if it had been completely converted into creatine, the change could not have been clearly demonstrated from the estimations of tissue creatine. Similar circumstances, we believe, explain also the failure of Mellanby (14) and Baumann and Hines (15) to show a definite increase in muscle creatine, following the administration of guanidoacetic acid.

The results seem to indicate that the kidneys may have played a prime rôle as a site of creatine formation in our experiments; yet it is not assumed that this occurs under normal conditions of metabolism. Although the evidence is fragmentary, it is not inconceivable that guanidoacetic acid may be formed at a fairly constant rate, that most of it is converted into creatine in muscle and of that which escapes and reaches the kidney a part is methylated and the rest excreted unchanged. This is an extension of Weber's conception (6) that the occurrence of guanidoacetic acid in urine represents an overflow phenomenon of an intermediate metabolic product formed in excess of the needs of the body. We have confirmed Weber's observation (16) concerning the occurrence of this constituent in the urine of normal men and women. That guanidoacetic acid may prove of considerable interest is indicated by its augmented excretion in both clinical and experimental hyperthyroidism and diminished output in hypothyroidism (unpublished data).

Suggestive as the present results may seem that guanidoacetic acid is the immediate precursor of creatine, it is realized that certain fundamental questions must be answered before this rela-

tion is firmly established. It must be shown, for example, that a source of guanidoacetic acid exists in the organism and that it is produced in amounts more than sufficient to account for all the creatine and creatinine produced in metabolism. Because of the predominance of creatine in muscle it will be of interest to determine whether this is not the principal site of guanidoacetic acid methylation.

SUMMARY

Creatine fed to rats in amounts of 50 to 100 mg. produced a slight, though transitory increase of its content in muscle. No definite change was observed in the myocardium. In the liver the concentration increased invariably, often as much as 400 to 500 per cent, returning to normal in 12 to 24 hours. In the kidney the change was equally striking. Analysis of the tissues for guanidoacetic acid revealed no departure from the normal.

Guanidoacetic acid was not found in the heart, muscle, and liver of normal rats, but traces were present in the kidneys and alimentary tract, including its contents.

At intervals following the administration of guanidoacetic acid, its distribution was determined. Of the amount absorbed, considerable quantities appeared in the liver and kidneys. Although its presence in muscle and heart was definite, the concentration was insufficient for quantitative estimation. As much as 50 per cent was excreted in the urine unchanged. These findings were accompanied by a definite increase of creatine in the kidneys (and urine), suggesting that methylation of the guanidoacetic acid may have occurred in the kidneys. However, it is not assumed that muscle may not be the principal site of guanidoacetic acid methylation in normal metabolism. In view of the occurrence of guanidoacetic acid in large amounts in the liver and the failure to show an increase in creatine, it is surmised that the liver plays an insignificant rôle, if any, in creatine production.

In the glycine-fed rats, the muscle did not show even the slight or moderate changes observed in the other groups. There was no increase in creatine in the liver and heart, and the change in the kidneys was more or less commensurate with the slightly augmented creatine excretion under these conditions. As regards the occurrence of guanidoacetic acid, there was essentially no departure from the normal.

The results of the present study furnish additional evidence for the conversion of guanidoacetic acid into creatine, but do not support the contention that glycine is a precursor of creatine.

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THE PARTITION OF REDUCED ASCORBIC ACID IN BLOOD

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It has been shown by several observers (1-5) that the titration method for the estimation of ascorbic acid may be applied to whole blood and plasma as well as to other animal tissues. Mirsky, Swadesh, and Soskin (4) reported values for the total ascorbic acid content of whole blood ranging between 1.11 and 2.88 mg. per cent. Farmer and Abt (5) determined the reduced ascorbic acid content of blood plasma and reported values of from 0.69 to 2.36 mg. per cent. The observation of an unusually high value for whole blood in a patient with leucemia led us to investigate the partition of ascorbic acid in blood. Determinations of the reduced ascorbic acid content of whole blood, plasma, packed red blood cells, and white blood cells were made on thirty specimens of blood obtained from a group of normal subjects and patients with a variety of pathologic conditions.

Venous blood was obtained from the subject either before breakfast or after a breakfast containing no appreciable source of vitamin C. Heparin or potassium oxalate was used as an anticoagulant. As a rule 50 cc. of blood were obtained for each analysis; smaller quantities were sufficient when the white blood cell count was very high. The whole blood was centrifuged at a speed of 2500 revolutions per minute for a period of 1 hour or longer. This resulted in the separation of the sample into three distinct layers, consisting of packed red blood cells, the buffy layer of packed white blood cells and platelets, and the supernatant plasma. Samples of plasma and red blood cells were removed

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and measured by means of a pipette. When the white blood cell count was normal, or but slightly elevated, the buffy layer was usually found as a closely packed coagulum which could be removed with forceps and transferred to a weighing bottle. The examination of a number of stained sections and differential counts of plasma suspensions of the buffy layer obtained in this manner showed that the sample was composed almost entirely of white blood cells; red blood cells were seldom present in excess of 5 per cent. By means of this procedure from 0.2 to 0.8 gm. of packed white blood cells could be obtained from 50 cc. of blood when the white blood cell count was normal. In patients with very high white blood cell counts, the creamy buffy layer was removed by means of a wide mouthed pipette.

To 2 cc. samples of whole blood, plasma, or packed red blood cells were added 2.5 cc. of 20 per cent trichloroacetic acid and 5.5 cc. of distilled water. The deproteinized filtrate was used for the estimation of ascorbic acid. The packed white blood cells were accurately weighed, transferred quantitatively to a mortar, and ground with fine sand. The resulting mixture was extracted by shaking for 5 minutes with 3 cc. of the 20 per cent acid mixture. After the addition of 9 cc. of distilled water, and thorough shaking for 10 additional minutes, the mixture was filtered.

The ascorbic acid content of the filtrates obtained as described above was determined by rapid titration against a standardized solution of 2,6-dichlorophenol indophenol, according to the method described by Birch, Harris, and Ray (6). The titrations were completed within 30 seconds and were always made in duplicate. Results were expressed as mg. of reduced ascorbic acid per 100 cc. of whole blood, plasma, and packed red blood cells and as mg. per 100 gm. of packed white blood cells.

Fujita and Iwatake (7) have observed a loss of ascorbic acid on standing in solutions of trichloroacetic acid and suggest the substitution of 2 per cent metaphosphoric acid for 5 per cent trichloroacetic acid in the extraction of tissues for the estimation of ascorbic acid. We have repeatedly demonstrated that the ascorbic acid content of trichloroacetic acid extracts of tissues and solutions of ascorbic acid remained constant for as long as 24 hours, when stored in the dark and kept cold and stoppered. The titration of extracts made with solutions containing metaphos-

phoric acid in excess of 1 per cent has proved unsatisfactory in our hands, principally owing to a marked frothing of the mixture during titration, making it difficult to reach a sharp end-point. At the suggestion of Dr. King¹ and Dr. Tressler,¹ an acid mixture containing 16 per cent of trichloroacetic acid and 4 per cent of metaphosphoric acid was substituted for 20 per cent of trichloroacetic acid in a number of experiments here recorded, but this seemed to offer no advantage over the use of trichloroacetic acid alone. When the precautions outlined by Ahmad (8) are followed, we have found trichloroacetic acid extracts satisfactory for the determination of the ascorbic acid content of blood and other tissues.

It is generally considered that the substance reacting with the indophenol reduction indicator under the prescribed conditions is ascorbic acid. It has been shown that the amounts of ascorbic acid determined by the titration method compare closely with those obtained by biologic assay (1). Cysteine, glutathione, and other substances which are known to reduce the indicator apparently do not interfere if the titration is carried out rapidly. Farmer and Abt (5) have shown that ascorbic acid may be quantitatively recovered from plasma deproteinized by means of trichloroacetic acid. Additional evidence of the identity of ascorbic acid and the reducing agent of animal tissues has been provided by the demonstration of the identity of the reduction velocities of trichloroacetic acid solutions of ascorbic acid and of comparable trichloroacetic acid extracts of rat intestine (9). The rate of reduction of the indicator by ascorbic acid was at least 350 times as fast as that by cysteine, glutathione, and material precipitated from tissue extracts by mercuric acetate.

The results of thirty experiments are shown in Table I. With the exception of the observations in patients with leucemia, the values for the ascorbic acid content of whole blood and plasma are similar to those reported by other investigators. Striking differences in the titration values of whole blood and plasma were observed only in those patients with high white blood cell counts. With but few exceptions, the ascorbic acid content of the red blood cells was found to be lower than that of whole blood or plasma, but the differences were not of great magnitude. In a few

¹ Personal communication.

instances, in patients with leucemia, unusually high values were obtained for plasma and red blood cells, as well as for whole

TABLE I
Ascorbic Acid Content of Whole Blood, Plasma, Red Blood Cells, and White Blood Cells

Subject	Diagnosis	White blood cells per c mm.	Whole blood	Plasma	Packed red blood cells	Packed white blood cells
			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.
C. S.	Pneumonia	7,500	0.66	0.70	0.90	8.06
H. J.	Normal	9,500	0.86	1.12	0.84	11.80
A. Da.	Peptic ulcer	5,650	0.86	0.88	0.86	7.65
A. G.	Polycythemia	8,200	0.86	1.02	0.90	10.1
A. De.	Cerebral hemorrhage	9,800	0.88	0.92	0.80	13.7
A. K.	" "	9,850	0.91	1.04	0.73	11.3
G. E.	Heart failure	11,400	0.95	1.00	0.72	11.15
D. W.	Peptic ulcer	9,800	0.96	0.96	0.78	10.8
M. P.	Polycythemia	13,200	1.03	1.20	1.02	12.0
A. G.	"	9,800	1.05	1.14	0.97	14.7
A. S.	Pneumonia	7,200	1.07	1.00	1.04	14.0
J. B.	Hypertension	9,750	1.07	1.16	1.33	11.85
J. D.	Cerebral hemorrhage	15,500	1.08	1.20	1.03	6.95
A. H.	Normal	6,800	1.10	1.10	0.94	8.65
M. P.	Polycythemia	11,400	1.10	1.35	1.32	8.47
T. B.	Tuberculosis	15,000	1.19	0.98	0.96	15.3
M. R.	Influenza	11,100	1.23	1.14	1.07	20.2
L. B.	Normal	8,300	1.27	1.19	1.19	13.15
R. L.	"		1.48	1.34	1.08	26.4
C. D.	Leucemia, myelogenous	96,000	1.66	1.52	1.04	8.12
A. K.	" lymphatic	22,000	1.90	1.95	4.25	17.0
C. S.	" "	28,700	2.20	1.63	1.74	8.20
M. T.	" myelogenous	17,600	2.58	1.20	0.97	31.0
J. W.	" "	117,200	3.23	2.25		30.0
"	" "	148,000	3.93	1.67	6.47	47.20
"	" "	152,000	3.96	3.75	2.50	14.20
S. B.	" lymphatic	590,000	5.25	0.91	0.95	75.0
E. G.	" myelogenous	463,000	5.45	1.14	0.98	15.35
"	" "	379,000	2.65		0.76	11.10
"	" "	103,000	1.72	1.06	0.95	19.4

blood. It is probable that incomplete separation of the white blood cells was responsible for these high values.

The ascorbic acid content of the packed white blood cells

varied widely but was consistently and significantly higher than that of any of the other fractions, ranging from 6.95 to 75 mg. per cent. In general, somewhat higher values were observed in patients with leucemia than in those with normal or but slightly elevated white blood cell counts.

Although the concentration of ascorbic acid in the leucocytes was many times that in the red blood cells or plasma, the actual amount contained in the white blood cells was relatively small, except in the patients with leucemia. In subjects with normal white blood cell counts, the leucocytes comprise less than 1 per cent of the total blood volume, so that high concentrations of ascorbic acid in the white blood cells have but little effect on the values obtained in whole blood. With very high white blood cell counts, however, the effect of the ascorbic acid content of the leucocytes on the values observed in whole blood becomes of significance. For example, the first determination in the case of E. G. was made at a time when the white blood cell count was 463,000 per c.mm. The white blood cells made up 27 per cent of the whole blood volume, as determined by hematocrit. The white blood cell hematocrit at the time of the second and third determinations in this patient was 18 per cent and 7 per cent, respectively. It is apparent that the unusually high values observed in the whole blood of the patients with leucemia were due to the preponderance of leucocytes.

The number of observations is too small to permit statistical treatment or correlation of the results with the various disease conditions or with the vitamin intake of the individual subjects. In general, however, the values for whole blood and plasma corresponded to the previous vitamin C intake of the non-leucemic subjects. The lowest values for whole blood and plasma were observed in subject C. S. who had taken no antiscorbutic foods for several months. The highest values in the subjects without marked elevation of the white blood cell count were observed in individuals with good vitamin C intake. There was no apparent correlation of the concentration of ascorbic acid in the leucocytes with that of the whole blood, plasma, or red blood cells.

SUMMARY

The reduced ascorbic acid content of whole blood, plasma, red blood cells, and white blood cells was determined by titration of

trichloroacetic acid extracts with the indophenol reduction indicator. The concentration of ascorbic acid in the white blood cells was found to be much higher than that of the other fractions. Unusually high values observed in the whole blood of subjects with leucemia were shown to be due to the preponderance of leucocytes in such samples.

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ADDITIONAL OBSERVATIONS ON THE ANEMIA CAUSED BY DEAMINIZED CASEIN*

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Steudel (1) demonstrated that deaminized casein is inadequate as a source of protein, and Hogan and Ritchie (2) observed that it may cause the development of anemia in animals that consume it. Gelatin and gliadin together form a complete protein mixture, but, when deaminized casein was included in the combination, anemia was the inevitable result. If, however, deaminized casein was combined with untreated casein, anemia did not develop and the animals grew normally. It was decided, therefore, to extend these studies to other proteins and to other substances that offered promise of accelerating or retarding the anemic condition.

A notable contribution on this type of anemia has been published by Smith and Stohlman (3) who reported that they had confirmed our more important observations. They also included a description of the morphological changes that occur in the red cells. They observed that the biological value of deaminized casein was not increased by the addition of amino acids, and the anemia was not relieved by the injection of liver extracts. If deaminized casein was treated with alkali, it no longer produced anemia, though it was still toxic and inhibited growth in young rats. These observations are in accord with our experience. The points on which we are not in entire agreement will be mentioned later.

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 462.

EXPERIMENTAL

Albino rats were used in these investigations. In order to shorten the experimental periods it was found advantageous to make the animals anemic before the experimental periods began, and then use the curative rather than the prophylactic technique. The anemia was first produced by limiting the animals to a milk diet, as described by Elvehjem and Kemmerer (4), until the red blood cell count had fallen to approximately 3 million per c.mm. The animals were then segregated in individual cages and put on the experimental rations. The cages were of the round, galvanized metal type, with screen floors, 2 meshes to the inch.

The rations used were of the conventional simplified type, and the approximate composition is as follows: milk fat 12.5, agar 2, salts 4 (5), cod liver oil 2, starch to make 100, protein 10 to 25. In later descriptions of individual rations the percentages of the first five constituents will be omitted as the first four remain constant and the percentage of starch is readily calculated. At first a mixture of tikitiki¹ and liver extract² was used as a source of the vitamin B complex, supplied separately. Each of these vitamin B carriers supplied 250 mg. of dry matter daily to each rat. Later 2 per cent of a water extract of yeast was included in the ration because some rats would not consume all of the tikitiki-liver extract mixture. In some of our more recent work 6 per cent of dried yeast was used as a source of the vitamin B complex instead of the water extract.

Minimum Amount of Casein That Prevents Anemia—In this extension of our investigations it seemed desirable to determine the minimum amount of casein that is required to protect against anemia. In our first efforts the casein was supplied separately as a supplement to Ration 1919, deaminized casein 10, gelatin 10, water extract of yeast 2. The smallest allowance of the untreated casein was 100 mg. daily and this amount was only slightly effective. The red cell count declined, although there was some slight gain in body weight. A dose of 200 mg. daily had considerably more activity, though the number of red cells increased very slowly. The initial count was 2.6 million per c.mm. and in 10 weeks this had increased to only 4.7 million. However, there were consider-

¹ Prepared in this laboratory by the method of Wells (6).

² Donated by Dr. David Klein of The Wilson Laboratories, Chicago.

able gains in weight, although the rate of growth was far below normal. Strangely enough a supplement of 400 mg. daily was not much more effective than 200 mg. There was a slight irregular gain in the number of red cells, also slight though rather consistent gains in body weight. Because of the large quantities required to protect against anemia, it was then decided to include

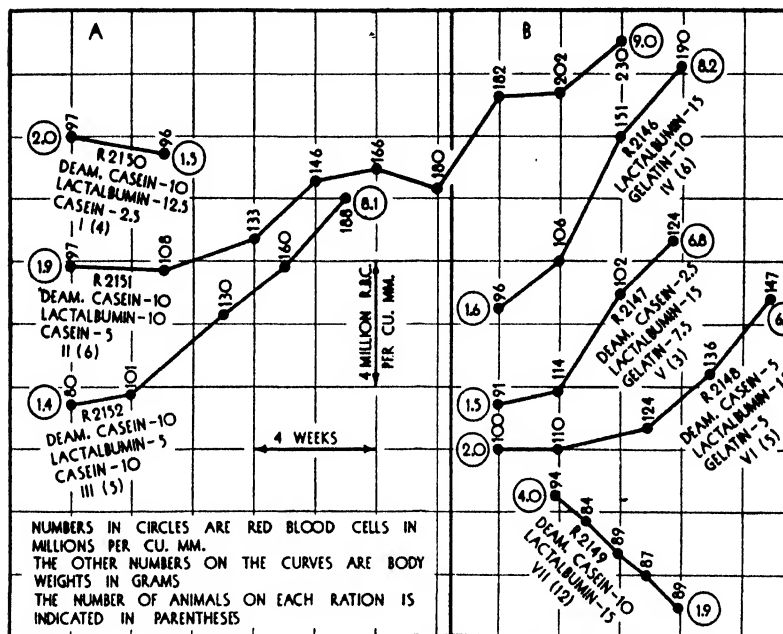


FIG. 1. The amount of untreated casein required to prevent anemia due to deaminized casein falls between 2.5 and 5 per cent of the ration used (Curves I to III). The amount of deaminized casein required to produce anemia falls between 5 and 10 per cent of the ration used (Curves IV to VII).

the casein in the original mixture (Curves I to III, Fig. 1, A). All the rations contained 10 per cent of deaminized casein and 2 per cent of the water extract of yeast. The percentages of casein used were 2.5, 5.0, and 10.0. In addition there was enough lactalbumin to make the total protein content 25 per cent. It developed at once that 2.5 per cent of casein is insufficient. With 5 per cent the animals became heavier and the red cell counts

increased consistently until they had attained a normal number. When the ration contained 10 per cent of casein, the animals grew rapidly and the red cell count quickly attained a high level. This would seem to show that at least when the ration contains 10 per cent of deaminized casein the protective level of ordinary casein lies somewhere between 2.5 and 5 per cent.

This protective action of casein is the only important respect in which our observations are at variance with those of Smith and Stohman. Their Ration 6 contained 10 per cent of deaminized casein and 15 per cent of casein. The animals that received this diet lost weight, and their red cells showed the abnormalities characteristic of this type of anemia. However, the lowest count taken was 6.56 million per c.mm., which would indicate that the destruction of erythrocytes had been greatly retarded. Our Ration 2152 (Curve III, Fig. 1) is not markedly different from their Ration 6 but it supported a satisfactory rate of growth and permitted rapid regeneration of red blood cells. We have no positive explanation of this divergence, but assume that it is due to differences in the methods of preparing the casein.

Minimum Amount of Deaminized Casein That Produces Anemia—It also seemed essential to determine the minimum amount of deaminized casein that is required to produce anemia. The basal diet for these studies contained 10 per cent of gelatin and 15 per cent of lactalbumin. When this ration was supplied to anemic rats, the red cells were rapidly regenerated and the rate of growth was satisfactory. When deaminized casein was included in the ration, it was substituted for an equal amount of gelatin (Curves IV to VII, Fig. 1, B). The lowest amount of deaminized casein was 2.5 per cent, then 5, and lastly the total amount was 10 per cent, thus replacing all of the gelatin. As would be expected from earlier observations, it was demonstrated that the ration containing 10 per cent of the deaminized casein, Ration 2149, was very far from being complete. There was a rapid decline in the number of red cells and there was a slight loss in body weight. When the ration contained 5 per cent of deaminized casein, the animals gained slowly but steadily in weight, and the red cells were regenerated at a rapidly increasing rate, until a normal count had been attained. When the ration contained 2.5 per cent of deaminized casein, there was some evidence that the red cells were regenerated somewhat more rapidly than at the 5 per

cent level, but the animals did not gain any more rapidly in body weight. The critical level of the deaminized casein seems, therefore, to lie somewhere between 5 and 10 per cent.

Gelatin and ovalbumin have been deaminized by slight modifications of the method used with casein. It seems worthy of record that their anemic activity is of the same order of magnitude as that of deaminized casein.

Deaminized Amino Acids—It had been assumed that a reaction product of nitrous acid and some amino acid was responsible for the injurious effects of deaminized casein, but all attempts to identify such a substance had failed. It seemed possible that, if the amino acids themselves were deaminized, the active agent might be obtained as a free compound, and that its isolation and identification would be greatly facilitated. The procedure was as follows: 250 gm. of casein, 745 gm. of concentrated sulfuric acid, and 1500 gm. of water were digested on a sand bath for 28 hours. The resulting solution was then diluted to 6 liters and enough barium hydroxide was added to neutralize three-fourths of the acid. The barium sulfate was filtered off and washed thoroughly with hot water. The filtrate and washings were then concentrated to 1500 cc. on a steam bath. 200 gm. of barium nitrite, dissolved in 500 cc. of water, were added slowly to the amino acid mixture, with constant stirring. This process required 2 hours. After standing overnight, barium hydroxide was added until the solution contained only a very slight excess of sulfuric acid. The barium sulfate was filtered off, washed thoroughly with hot water, and the combined filtrate and washings evaporated almost to dryness on a steam bath. The residue was gum-like and difficult to manipulate, so 50 gm. of calcium hydroxide, suspended in a little water, were added to the deaminized mixture and it was again evaporated to dryness. The calcium salts formed a friable mixture that could be ground to a powder and easily incorporated in the ration. From 250 gm. of casein the yield was 225 gm. The ration contained 10 per cent of the deaminized amino acids and 15 per cent of lactalbumin. This diet had no depressing effect upon the red blood cells, for it was observed that they were regenerated at almost the normal rate. Apparently, however, these deaminized amino acids were toxic, as was shown by the more or less rapid loss in body weight.

Miscellaneous Group of Substances Failing to Prevent Anemia—

As the next step in the search for some remedial agent it was decided to examine a number of miscellaneous products for their curative action. The basal ration used, Ration 2149, was the same in all cases. The various substances investigated included milk, egg yolk, the water extract of yeast, wheat germ oil, and a number of animal tissues. These were muscle, stomach, and liver in amounts ranging from 100 to 400 mg. daily. In no case could any important curative action be observed.

It is well known that iron and copper are essential for hemoglobin formation, so in a few cases additional amounts were supplied the experimental animals. These adjuvants, 0.5 mg. of iron, and 0.05 mg. of copper daily per animal were entirely ineffective.

Relation of Nitroso Compounds to Anemia—As has been mentioned before, there is practically no information as to the reactions that occur when proteins are treated with nitrous acid. However, the suggestion has been made that nitroso compounds are formed. It seemed desirable, therefore, to investigate the possibility that the toxic action of deaminized casein may be due to some such substance. Two of these compounds have been investigated, nitrosobenzene³ and the sodium salt of *p*-nitrosophenol.³ The basal diet was Ration 2221, which contains 15 per cent lactalbumin and 2 per cent of the water extract of yeast. The amount of nitroso compounds was 0.1 per cent of the basal ration. The nitrosophenol had little or no effect. The animals grew rapidly and the red blood cells were regenerated at the normal rate. The nitrosobenzene is apparently much more toxic. The rate of growth was depressed and the regeneration of red blood cells was markedly retarded. It is improbable that nitroso compounds of the type investigated are present in deaminized casein, so it is hoped that similar trials may be conducted with compounds that have the nitroso group attached at a peptide linkage.

Lactalbumin and Ovalbumin—It has been mentioned that rats developed anemia and died when they consumed rations that contained deaminized casein, gelatin, and gliadin, but remained normal and grew at the usual rate when untreated casein replaced the gelatin and gliadin. In the hope of obtaining some indication of the reason for this contrast in behavior, these studies have included two other proteins, lactalbumin and ovalbumin.

³ Purchased from the Eastman Kodak Company, Rochester, New York.

In the preparation of lactalbumin the casein was precipitated from skim milk with acid, and filtered off. The filtrate was then brought to the boiling point with live steam, and, after a suitable adjustment of the hydrogen ion concentration, the lactalbumin separated readily. This was filtered off on cloth, washed twice with hot water, dried, and ground. This material was altogether ineffective in preventing anemia. It seemed possible that the long exposure to high temperatures may have destroyed the protective agent, so another lot was prepared by salting-out. After the casein had been removed, the whey was saturated with sodium chloride, acidified with hydrochloric acid, and the precipitate filtered off. The precipitated lactalbumin was washed with dilute ethyl alcohol until practically all the salt was removed, then dried, and ground. This preparation was likewise ineffective in healing anemia due to deaminized casein. An attempt was made to replace our laboratory preparation with a commercial product, but healing followed invariably. We have no satisfactory explanation of this variability, but it may be due to some apparently inconsequential difference in the method of preparation.

The ovalbumin was a commercial dried product, but in most of our trials it was reprecipitated with a view to obtaining a more uniform preparation. The dried material was dissolved in warm water, acidified, and coagulated by heating the solution with live steam. The coagulum was filtered off, washed twice thoroughly with hot water, dried, and ground. Our results with this source of protein have been decidedly inconsistent. When combined with deaminized casein it usually failed to prevent anemia, but some preparations brought about prompt healing. This discrepancy has not been explained. When the original commercial product is combined with deaminized casein, healing of anemia invariably follows. A comparison of the two types of ovalbumin is shown in Curves I and II of Fig. 2, A.

Ovovitellin—At this stage the contrast between casein on one hand and lactalbumin or ovalbumin on the other was very striking. Casein is a phosphoprotein, and at the time that seemed to be the most important difference. This suggested that other phosphoproteins might behave in the same manner as casein, so ovovitellin was prepared from egg yolk by the method of Calvery and White (7). Only limited quantities were available at the time so it was

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offered as a supplement to Ration 2149. In the quantities supplied, 200 and 400 mg. daily, it was completely ineffective. Ra-

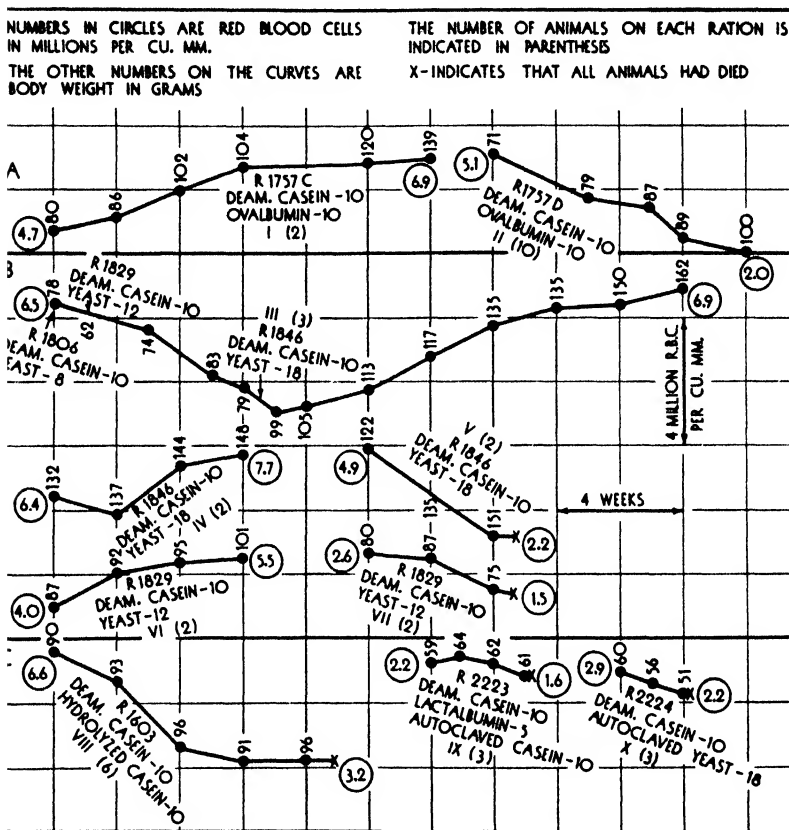


FIG. 2. Commercial ovalbumin (Curve I) prevents deaminized casein anemia, but most laboratory preparations (Curve II) do not. Large amounts of yeast (Curves III to VII) are at least partly effective, but their action is less consistent. Hydrolyzed casein (Curve VIII), autoclaved casein (Curve IX), and autoclaved yeast (Curve X) are ineffective.

tion 2149 contains deaminized casein 10, lactalbumin 15, water extract of yeast 2.

Casein Extracts—The observations just described rendered it improbable that an amino acid is responsible for the protective

action of casein, so other possibilities were considered. If the active agent were adsorbed by casein, one would suppose it could be removed by an appropriate reagent. Casein was therefore extracted with a variety of solvents including water, dilute acid, also neutral, acid, and alkaline alcohol; but none of these preparations had the slightest activity, so this method of attack was abandoned.

Dried Yeast—It was hoped that useful extracts might be prepared from some other source, so yeast was examined with that end in view. However, before making any attempt to prepare an extract, yeast itself was combined with deaminized casein to demonstrate whether or not it contains the antianemic agent. The first ration, No. 1806, contained 10 parts of deaminized casein and 8 parts of dried yeast. The animals declined rapidly in weight and so the amount of yeast was increased to 12 per cent (Ration 1829). When this was done the loss in weight was arrested, but the red cell counts continued to fall until they attained a level of about 3 million. At this point the amount of yeast was increased to 18 per cent (Ration 1846) and the animals began gaining in weight at a little less than the normal rate and new red cells were slowly regenerated. In a period of 10 weeks the number of red corpuscles was practically normal. Additional details are shown in Curve III of Fig. 2, B.

It developed later that in the response to yeast there are minor inconsistencies which are not easily explained. Two groups (Curves IV and V, Fig. 2, B) were started on Ration 1846, but there were some differences in the initial levels of red blood cells. One group began with an average count of 6.4 million, and this increased slowly to 7.7, accompanied by a slight gain in weight. The other began with an average count of 4.9 million but though there was a significant gain in weight the red cell count declined consistently and the rats died. Two other groups (Curves VI and VII, Fig. 2, B) were started on Ration 1829, which contains 10 per cent of deaminized casein and 12 per cent of yeast. One group began with an average count of 4.0 million, and this gradually rose to 5.5 million, with concomitant gains in body weight. The other began with an average count of 2.6 million, but there were declines both in red cell counts and in body weights, and the animals died. Regardless of these inconsistencies, however, it is

certain that yeast has some protective action against this type of anemia. All attempts made thus far have failed to identify the active agent with any particular chemical group.

These observations are in substantial agreement with those of Smith and Stohlgan, who included 12 per cent of yeast in their Ration 4. In our opinion their animals would have been protected from anemia still more completely if the amount of yeast in their rations had been raised to a markedly higher level.

Water Extract of Yeast—As mentioned previously, one reason why yeast was chosen for examination was the possibility that a useful extract might be prepared from it. Accordingly, a water extract of yeast was prepared, which is known to be active as a source of the vitamin B complex, and 10 per cent of this extract was included in a ration with 10 per cent of deaminized casein. In a period of 3 weeks the red cell count declined from 2 to 1 million, but there were in that time no marked changes in body weight.

Stability of Antianemic Factor—In the hope of gaining some suggestions as to the factors responsible for anemia it was decided to test the thermostability of substances that have a protective action. Accordingly, both yeast and casein were heated in an autoclave for 5 hours at 135°. When these materials were substituted for the untreated preparations (Curves IX and X, Fig. 2, C) it was found that their antianemic activity had entirely disappeared. It has been shown by Hogan (8) that autoclaving does not seriously impair the nutritional properties of protein, so it is reasonably certain that the antianemic potency of casein is not due to the presence of some unique amino acid. Whatever the source of this activity may be, it is destroyed by high temperatures.

Hydrolyzed Casein—It was originally supposed that the protective action of casein was due to some special amino acid that it contained. It was hoped that casein might be hydrolyzed and then the active amino acid could be isolated and identified. Before this was attempted, however, the hydrolyzed casein was combined with deaminized casein as a control to determine whether the amino acid mixture was as effective as the original casein. It developed at once (Curve VIII, Fig. 2, C) that the protective action of the original product had almost completely disappeared. The number of red cells declined, although there was little change

in the body weight of the experimental animals. In view of later observations, however, there is no reason to suppose the process of hydrolysis itself had destroyed the antianemic agent. As will be shown later the destruction may have been due to other causes.

Hydrolyzed Deaminized Casein—Before we published our first paper (2) we had hydrolyzed deaminized casein, in the hope that the anemic agent could be recovered from the products of hydrolysis. Several such hydrolysates were prepared, but only one gave any evidence of anemic activity, and that line of attack was temporarily abandoned. The deaminized casein had been hydrolyzed with sulfuric acid and then the acid removed with barium. It had been observed, however, that even after thorough washing with hot water the barium sulfate precipitate still contained a large amount of nitrogen. It had been our practice to remove the sulfuric acid by rapidly adding barium hydroxide until the solution was alkaline to litmus. The excess barium was then removed by cautiously adding sulfuric acid. Our first hypothesis was, the substance that produces anemia had been precipitated as a barium salt. It was decided, therefore, to make another attempt to recover the toxic agent by changing slightly the method of removing the sulfuric acid. The procedure now used is as follows:

500 gm. of the deaminized casein, 1490 gm. of concentrated sulfuric acid, and 3000 gm. of water are boiled on a sand bath, under a reflux condenser, for 24 to 26 hours. After cooling, barium hydroxide is added cautiously until the mixture is barely acid to brom-phenol blue, and the barium sulfate is filtered off. The precipitate is washed five or six times with hot water, and the filtrate and washings combined and evaporated almost to dryness on the water bath. The residue is gum-like in consistency, so to facilitate handling just enough calcium hydroxide is added to make it friable in texture. When this product is suspended in water it is still acid to brom-phenol blue, but alkaline to thymol blue. This material is substituted for the deaminized casein of Ration 2149. Two such preparations have been studied up to date. Four rats received Preparation I, and they declined and died in about the same time as would be expected on deaminized casein. The average weights and red cell counts of these animals are shown in Curve V of Fig. 3. Four others received Preparation II, but their response was somewhat more variable. One of them

it seems to be only slightly, or not at all, less effective than casein itself. This material is substituted for the casein of Ration 2152. Five rats have received this diet, and they recovered from anemia promptly, and grew at a satisfactory rate. Their response is shown in Curve VI of Fig. 3.

Our studies of the hydrolysates of deaminized casein and of casein will be continued, in an effort to isolate the active agents and to define their properties more precisely.

SUMMARY

1. With the experimental conditions as described, the approximate amount of casein required to prevent anemia was 5 per cent of the ration. The minimum amount of deaminized casein required to produce anemia lies between 5 and 10 per cent of the ration.

2. A miscellaneous group of substances including milk, egg yolk, wheat germ oil, ventriculin, muscle, liver, and stomach were tested for antianemic activity. At the levels tested, from 100 to 400 mg. of dry matter daily, they were ineffective.

3. Two nitroso compounds were tested to see whether they would cause anemia. Both were toxic. *p*-Nitrosophenol had no effect on the rate at which red blood cells were formed, but the rate of formation was retarded by nitrosobenzene.

4. Lactalbumin that was prepared in the laboratory does not prevent this type of anemia. Variable results were obtained with a laboratory preparation of ovalbumin. Commercial preparations of both proteins prevent anemia consistently.

5. Dried yeast, 18 per cent, confers protection against the anemia caused by deaminized casein.

6. An attempt to extract the active agent of yeast with water was not successful.

7. Autoclaved casein and autoclaved yeast do not overcome the anemia caused by deaminized casein.

8. When deaminized casein was hydrolyzed with 25 per cent sulfuric acid at atmospheric pressure, the active agent was recovered in the hydrolysate. When casein was hydrolyzed in a similar manner, the antianemic agent was likewise recovered in the hydrolysate.

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CHEMICAL STUDIES ON THE VIRUS OF TOBACCO MOSAIC

VII. AN IMPROVED METHOD FOR THE PREPARATION OF CRYSTALLINE TOBACCO MOSAIC VIRUS PROTEIN*

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A method for the isolation, from mosaic-diseased Turkish tobacco plants, of a crystalline protein possessing the properties of tobacco mosaic virus has been described (1, 2). The method consists essentially in precipitation of the globulin fraction from a dilute disodium phosphate extract of frozen, macerated, infected plants with ammonium sulfate, and the removal of most of the remaining colored materials by treatment with lead subacetate and celite. The yield of crystalline protein by this method amounts to about 40 per cent of the crude twice precipitated globulin fraction; a loss of about 50 per cent occurs during the single treatment with lead subacetate. It was found that practically all of the virus protein could be precipitated by simply doubling the amount of lead subacetate used. Because of the large loss of active protein involved in the successful use of this method, and because of the possibility of sustaining an even greater loss when an excess of lead subacetate was used, it seemed desirable to determine whether or not lead subacetate could be eliminated from the purification process. This was found to be possible and an improved method, by means of which the yield of crystalline protein from crude globulin was increased to about 80 per cent, has been devised. The method is described in this paper.

* For Paper VI of this series see (1).

EXPERIMENTAL

The new method involves the use of ammonium sulfate, celite (Hyflo Standard-cel), and calcium oxide, and a starting material consisting of the dark brown crude globulin preparation previously described (1). This starting material is the globulin fraction in a dilute disodium phosphate extract of frozen, macerated, mosaic-diseased Turkish tobacco plants, which has been filtered through celite and precipitated twice by means of ammonium sulfate. Previously the extracts were adjusted to about pH 5 before precipitation of the protein with ammonium sulfate. This adjustment, involving the use of considerable acid, has been found unnecessary, and the globulin is now precipitated at about pH 7 or 8 and then taken into solution with 0.1 M sodium phosphate at pH 7 or 8. Although the filtrate from the second precipitation with ammonium sulfate is usually deeply colored, the filtrate from the third and additional precipitations with ammonium sulfate are usually light colored or colorless. Therefore, except in the case of occasional very dark colored preparations, only two precipitations with ammonium sulfate are now used in the preparation of the starting material.

A representative experiment in which crystalline tobacco mosaic virus protein was prepared by means of the new method is described in this and the following paragraph. Eleven rapidly growing Turkish tobacco plants in 6 inch pots in a greenhouse were inoculated by rubbing over the leaves 2 cc. of a solution of 2 mg. of crystalline tobacco mosaic virus protein in 0.1 M sodium phosphate at pH 7 on April 14, 1936, when the plants were 2 to 4 inches high. 4 weeks later they were cut, frozen, put through a meat grinder, and the pulp, which weighed 1360 gm., was extracted twice with 0.1 M sodium phosphate at pH 7. The first extract consisted of 1000 cc. and contained 1.2 mg. of total nitrogen and 0.68 mg. of protein nitrogen per cc. The second extract of 950 cc. contained 0.2 mg. of total nitrogen and 0.14 mg. of protein nitrogen per cc. The two extracts were combined, 585 gm. of ammonium sulfate were added, and the precipitated globulin was collected by means of gravity filtration, folded Carl Schleicher and Schüll No. 1450½ filter paper being used. The virus activity of the filtrate and of the precipitate after this and subsequent treatments was determined by means of infectivity

tests on leaves of *Nicotiana glutinosa*, L., with the half leaf method (3-5). The preparations used for these tests were first dialyzed against distilled water and then adjusted so that they contained 0.1 M phosphate at about pH 7 before inoculation to plants. Protein nitrogen analyses were made on this and subsequent filtrates in order to determine the loss of protein during each treatment.

The precipitate obtained on filtration, consisting of 3.9 gm. of protein, was dissolved in 500 cc. of 0.1 M phosphate solution at pH 7 and filtered, with suction, through a thin (about 0.5 cm.) layer of celite on a Buchner funnel. The celite filter cake was washed with 100 cc. of 0.1 M phosphate at pH 7 and the protein in the 600 cc. of filtrate was precipitated by the addition of 120 gm. of ammonium sulfate. The precipitated protein was collected on folded filter paper and then dissolved in 500 cc. of 0.1 M phosphate at pH 7. To this solution, which was found to contain 3 gm. of protein, were added 40 gm. of ammonium sulfate, and the slightly turbid solution was filtered, with suction, through a thin layer of celite on a Buchner funnel. In other experiments the amount of ammonium sulfate required to produce a slightly turbid solution varied somewhat depending on the protein concentration, but usually was found to be about 8 to 11 per cent by weight. The celite filter cake was washed with 100 cc. of 8 per cent ammonium sulfate solution and this filtrate added to the main portion. To the clear, brown-colored filtrate, which was found to contain 2.6 gm. of protein, were added 72 gm. of ammonium sulfate in order to bring the ammonium sulfate concentration up to 20 per cent by weight; and the precipitated protein was removed by filtration with a thin layer of celite on a Buchner funnel. All of the virus activity was retained on the celite and much colored matter was lost in the filtrate. Most of the active protein on the celite filter cake was removed by extracting once with 400 cc. and twice with 150 cc. of 0.1 M phosphate solution at pH 7. The combined extracts, which were opalescent and light brown in color, and contained 2.5 gm. of protein, were adjusted to pH 4.5 by the addition of 6 cc. of 3 M H_2SO_4 and filtered through a thin layer of celite. All of the virus activity was retained on the celite filter cake, and colored material and a small amount of inactive protein were lost in the filtrate. The celite

filter cake containing the active protein was suspended in 300 cc. of water in order to make about a 1 per cent protein suspension, and the hydrogen ion concentration was adjusted to pH 8 by the addition of 7 cc. of an aqueous suspension of 5 per cent calcium oxide. The suspension was then filtered with suction on a Buchner funnel, and the celite filter cake was extracted three times with 150 cc. of water at pH 8. The four filtrates were combined and gave a practically colorless opalescent solution. The protein in this solution was crystallized by the addition of, first, 75 gm. of solid ammonium sulfate, then 6 cc. of a solution of 5 per cent glacial acetic acid in 0.5 saturated ammonium sulfate, and finally 20 cc. of saturated ammonium sulfate solution. The yield of once crystallized protein was 2.3 gm. or 77 per cent based on the crude twice precipitated globulin.

No differences were found in the properties of the crystalline protein prepared by the method just described and the properties of the protein prepared by the method involving the use of lead subacetate. The virus activity, optical rotation, nitrogen content, isoelectric point, and serological properties of protein prepared by the two methods were the same within the experimental errors. Furthermore, crystals of protein prepared by the two methods were indistinguishable under the microscope.

DISCUSSION

The separate treatments that have been embodied in the improved method are the ones that, during the past year, have proved useful in the separation of colored matter and extraneous protein from the virus protein. Because of the variation in starting material, these treatments may be modified considerably, and in some instances certain treatments may be entirely omitted. Plants inoculated while quite small and subsequently grown in a greenhouse for at least 4 weeks have been found to yield the best starting material. Such plants contain a minimum of pigment and extraneous protein and occasionally yield protein which may be crystallized after only two precipitations with ammonium sulfate, thus making unnecessary the adsorption on celite at pH 4.5 and the treatment with CaO. Plants that are inoculated when fairly large contain extraneous protein, and very old greenhouse plants or plants grown outside in a field contain pigment that is very difficult to separate from the active protein fraction.

The treatment with CaO has been used quite extensively in the purification of virus protein, and has proved especially useful in removing, from a slightly colored solution of active protein, the last portions of colored materials which are usually quite difficult to remove, only ammonium sulfate and celite being used. The exact method of treatment may be varied considerably. Solutions containing from about 0.5 per cent to about 2 per cent of protein may be used. It is not necessary, for example, to use the suspension of protein and celite obtained from the filtration at pH 4.5 as starting material for the CaO treatment. The suspension of CaO may be added directly to solutions containing from about 0.5 per cent to about 2 per cent of active protein at hydrogen ion concentrations ranging from about pH 4.5 to 7. The amount of CaO suspension required to bring the hydrogen ion concentration to about pH 8 depends on the acidity of the protein solution to be treated. The hydrogen ion concentration must not be allowed to go above about pH 8.5 on addition of the suspension of CaO, since at more alkaline reactions the protein tends to become denatured and there is loss of activity. Celite may be added to solutions already containing the calcium salt and the filtration carried out as usual, or, if it is desired, the solution may be filtered through a layer of celite on a Buchner funnel. When protein solutions are to be filtered through paper on a Buchner funnel with suction, rapid filtration may be obtained by first impregnating the paper with a small amount of celite.

Recent ultracentrifugal studies by Eriksson-Quensel and Svedberg (6) and by Wyckoff and Biscoe (7) indicate that wide changes of hydrogen ion concentration or prolonged treatment cause the virus protein to become non-homogeneous with respect to molecular weight. In order to retain the molecular homogeneity of the protein during its isolation and purification it is advisable, therefore, to subject it to no more variation of hydrogen ion concentration and to no more treatment than is absolutely necessary.

It is interesting to note that, in the experiment reported, the 2.3 gm. of virus protein which were isolated were produced in eleven plants that had been inoculated with a solution containing a total of only 2 mg. of active protein. It is known that most of the inoculum rubbed on a leaf remains on the surface of the leaf, and that only a very small portion actually enters the cells

to serve as infective material. If one assumes that about 0.001 of the protein in the inoculum actually gets into the plant, then it may be calculated that during the course of 4 weeks the protein introduced into the plant has been increased over a million times.

SUMMARY

An improved method for the preparation of crystalline tobacco mosaic virus protein, involving the use of ammonium sulfate, celite, and calcium oxide, and by means of which the yield from crude twice precipitated globulin has been increased to about 80 per cent, is described.

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FURTHER STUDIES ON THE CALCIUM CONTENT OF THE BODY IN RELATION TO THE CALCIUM AND PHOSPHORUS CONTENT OF THE FOOD

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Normal calcification in the growing and developing body clearly requires adequate intakes of both calcium and phosphorus. To what extent it is influenced also by the ratio of intakes of calcium and phosphorus when a liberal amount of each of these elements is supplied, has not been clear; and it was for the purpose of obtaining further light upon this question that the experiments here recorded were undertaken. For the sake of brevity we do not attempt a comprehensive review, but cite only the immediate background of the present experiments.

Sherman and MacLeod (1) made a systematic series of determinations of calcium in normal white rats of known nutritional history which were killed for analysis at definite age intervals, thus establishing quantitatively a scale of normal percentages of body calcium for such rats at different ages. Rat families of this same strain and of very similar nutritional histories have been continued in our colony for several years with practically no change in the rate of calcification as shown by analyses of representatives of different generations. Sherman and Booher (2) investigated the influence of graded intakes (0.16 to 0.50 per cent in the air-dry food mixture) of calcium upon the rate of increase of the percentage of calcium in the growing body.

The purpose of the present investigation was to determine whether increasing the calcium content of the diet still further (0.53 to 1.04 per cent) in diets essentially similar to those used by Sherman and Booher would induce more rapid normal calcification, first, when the phosphorus content of the diet was essentially

* Deceased.

the same (0.43 per cent) as that used in the earlier work of this laboratory, and secondly, when the phosphorus content was increased to 0.73 per cent. These experiments thus deal with the problem of optimal calcification as distinguished from the conditions of occurrence of rickets as studied simultaneously by Shohl and Wolbach (3).

EXPERIMENTAL

Young albino rats which had been reared to 28 to 30 days of age by mothers on a diet consisting of one-third whole milk powder and two-thirds ground whole wheat were thereafter raised to definite ages on diets which were similar except in their calcium and phosphorus contents. One series of animals received diets containing 0.43 per cent phosphorus, while the calcium contents were 0.53, 0.70, 0.84, or 1.04 per cent, respectively. A second series of animals received diets containing 0.73 per cent phosphorus, while the calcium contents were 0.55, 0.71, 0.89, or 1.05 per cent, respectively. The relative nutritional effect of these diets through a considerable portion of the life history of the animals was compared in terms of growth, calcium content of the bodies, and skeletal development as measured by body lengths at definite ages.

In all cases the animals were allowed to consume food and distilled water *ad libitum*. The calcium analyses were made according to the modified McCrudden method as previously used in this laboratory (1), the percentage of body calcium being expressed on the basis of the maximum live weight minus the weight of the contents of the digestive tract.

Series I—The four diets fed to animals of this series contained in common per 1000 gm. of diet the following constituents: 100 gm. of whole milk powder, 55 gm. of casein, 68 gm. of butter fat, and 16 gm. of sodium chloride. In addition the diets contained 8.5, 12.8, 16.8, and 21.0 gm. respectively, of calcium carbonate and a sufficient amount of ground whole wheat to make a total of 1000 gm. of food mixture in each case. These diets were analyzed and found to contain 0.43 per cent phosphorus and 0.53, 0.70, 0.84, and 1.04 per cent calcium respectively, with corresponding Ca:P ratios of 1.2, 1.6, 2.0, and 2.4, respectively. These diets were shown to be adequate in vitamin D value.

The animals reared on these diets to 300 days of age were caged

TABLE I

Calcium Contents of Bodies of Rats on Diets Containing 0.43 Per Cent Phosphorus and 0.53 to 1.04 Per Cent Calcium

	Age	Ca con- tent of diet	Ca:P ratio of diet	No. of cases	Aver- age net body weight	Total Ca in body	
		days per cent			gm.	gm.*	per cent*
Males	60	0 53	1 2	10	139	1.20 ± 0.16	0.87 ± 0.03
		0 70	1 6	4	117	1.05 ± 0.11	0.91 ± 0.03
		0 84	2 0	10	123	1.09 ± 0.13	0.89 ± 0.04
		1 04	2 4	4	96	0.87 ± 0 04	0.91 ± 0.03
Females	60	0 53	1 2	11	117	1.12 ± 0.12	0.96 ± 0.03
		0 70	1 6	4	93	0.95 ± 0 09	1.03 ± 0.02
		0 84	2 0	12	100	0.96 ± 0.11	0.97 ± 0.04
		1 04	2 4	4	88	0.86 ± 0 05	0.98 ± 0.04
Males	90	0 53	1 2	8	178	1.70 ± 0.29	0.96 ± 0.04
		0 70	1 6	4	183	1 83 ± 0.28	1 01 ± 0.04
		0 84	2 0	10	198	1.88 ± 0 23	0.96 ± 0.04
		1 04	2 4	5	170	1 62 ± 0.21	0.95 ± 0.03
Females	90	0 53	1 2	10	151	1 63 ± 0.15	1.09 ± 0.04
		0 70	1 6	4	148	1 66 ± 0 13	1.13 ± 0.04
		0 84	2 0	9	137	1 57 ± 0.21	1.16 ± 0.05
		1 04	2 4	4	133	1 49 ± 0.24	1.13 ± 0.03
Males	180	0 53	1 2	4	268	2.76 ± 0.34	1.03 ± 0.02
		0 70	1 6	4	261	2.82 ± 0 30	1.08 ± 0.04
		0 84	2 0	3	236	2.65 ± 0 35	1.14 ± 0.05
		1 04	2 4	3	225	2.40 ± 0 14	1 07 ± 0.03
Females	180	0 53	1 2	5	160	1.94 ± 0.21	1.21 ± 0.01
		0 70	1 6	4	153	1.95 ± 0.19	1.29 ± 0.07
		0 84	2 0	4	168	2.01 ± 0.26	1.20 ± 0.01
		1 04	2 4	4	162	2.01 ± 0.24	1.24 ± 0.03
Males	300	0 53	1 2	4	310	3.27 ± 0.23	1.06 ± 0.03
		0 70	1 6	4	300	3 31 ± 0.19	1.11 ± 0.03
		0 84	2 0	4	283	3.10 ± 0.23	1.10 ± 0.02
		1 04	2 4	4	283	3.12 ± 0 09	1.10 ± 0 04
Females	300	0 53	1 2	4	192	2.40 ± 0.29	1.26 ± 0.03
		0 70	1 6	4	200	2.69 ± 0.20	1.35 ± 0.02
		0 84	2 0	4	202	2 62 ± 0.18	1.30 ± 0.04
		1 04	2 4	4	201	2.68 ± 0.11	1.33 ± 0.05

* ± average deviation.

together in groups of four of the same sex. The animals reared to 60, 90, and 180 days of age were kept in smaller individual cages equipped with wide mesh screen bottoms.

The calcium contents of the bodies of rats reared to 60, 90, 180, and 300 days of age on the diets containing 0.43 per cent phosphorus and 0.53, 0.70, 0.84, or 1.04 per cent calcium, respectively, are shown in Table I. It is apparent from Table I that the animals on these different diets made essentially equal development. Their growth rates were all within a range that may be considered normal and there was no continuous trend of difference in growth corresponding to the graded differences in the calcium contents of the diets. Measurements of the body lengths of the rats which were reared on the four different diets and whose bodies were subsequently analyzed for calcium showed almost identical average body lengths at definite ages. It is apparent, therefore, that the calcium content of the body, the rate of growth, and the length of the body at definite ages, were not affected by the graded differences in the calcium contents of the food, nor by the Ca:P ratios of these four diets.

Series II—The general procedure for the experiments on the animals of this series was essentially the same as that described above in connection with Series I, except that the phosphorus content of the diets was increased to 0.73 per cent and the rats were reared to 60 and 90 days only. The four diets fed contained the same kinds and amounts of the constituents which were common to the diets of Series I. In order to raise the phosphorus level, 15.5 gm. of dicalcium phosphate were added to each of the food mixtures described above, while the adjustments of the calcium levels to approximately those of the first series were made by the addition of 0, 3.8, 7.8, and 12 gm., respectively, of calcium carbonate, and sufficient ground whole wheat to bring the total to 1000 gm. in each case. These diets on analysis yielded 0.73 per cent phosphorus and 0.55, 0.71, 0.89, and 1.05 per cent calcium, respectively. Their Ca:P ratios were therefore 0.7, 1.0, 1.2, and 1.4, respectively.

At 60 and 90 days of age both males and females showed very small increments in the calcium content of the body as the amounts of calcium in the diet increased from 0.55 to 1.05 per cent, corresponding to Ca:P ratios of 0.7 to 1.4. The analyses are sum-

marized in Table II. The results for the two series suggest that in a diet which is adequate in both calcium and phosphorus content the optimal Ca:P ratio during growth is about 1.2 or 1.5. It also appears that the Ca:P ratio is of minor efficacy in comparison with that of the provision of liberal quantities of both calcium and phosphorus. The rats of this second series did not show significant differences in rate of growth or body lengths at dif-

TABLE II

Calcium Contents of Bodies of Rats on Diets Containing 0.73 Per Cent Phosphorus and 0.55 to 1.05 Per Cent Calcium

	Age	Ca content of diet	Ca:P ratio of diet	No. of cases	Average net body weight	Total Ca in body	
		per cent			gm.	gm.*	per cent*
Males	60	0.55	0.7	5	140	1.20 ± 0.13	0.86 ± 0.02
		0.71	1.0	4	141	1.23 ± 0.12	0.88 ± 0.03
		0.89	1.2	5	142	1.30 ± 0.07	0.92 ± 0.02
		1.05	1.4	3	137	1.30 ± 0.04	0.95 ± 0.00
Females		0.55	0.7	5	118	1.14 ± 0.05	0.97 ± 0.02
		0.71	1.0	5	117	1.18 ± 0.06	1.00 ± 0.04
		0.89	1.2	5	117	1.19 ± 0.10	1.02 ± 0.03
		1.05	1.4	5	114	1.22 ± 0.11	1.07 ± 0.03
Males	90	0.55	0.7	4	202	1.81 ± 0.22	0.89 ± 0.02
		0.71	1.0	4	195	1.79 ± 0.22	0.92 ± 0.02
		0.89	1.2	4	199	1.92 ± 0.26	0.96 ± 0.02
		1.05	1.4	4	183	1.77 ± 0.23	0.95 ± 0.03
Females		0.55	0.7	4	144	1.52 ± 0.05	1.06 ± 0.02
		0.71	1.0	4	143	1.55 ± 0.11	1.09 ± 0.02
		0.89	1.2	4	148	1.68 ± 0.15	1.13 ± 0.02
		1.05	1.4	4	148	1.65 ± 0.06	1.12 ± 0.04

* ± average deviation.

ferent ages among themselves, nor were these two measurements for this series significantly different from those of the first series.

SUMMARY

Previous work has shown that, with a diet containing 0.42 to 0.43 per cent of phosphorus, progressive increases in the calcium content of the diet from 0.16 to 0.21, 0.33, and 0.50 per cent, respectively, with corresponding Ca:P ratios of 0.3, 0.5, 0.7, and

1.2, respectively, result in progressive increases in the rates of calcification of the animal body.

In the present investigation, further increases in the calcium content of essentially the same diet by graded amounts to 0.53, 0.70, 0.84, or 1.04 per cent, respectively, with consequent corresponding Ca:P ratios of 1.2, 1.6, 2.0, and 2.4, respectively, did not bring about any distinct increase in the rate of calcification of the body. When the phosphorus content of the diet was increased to 0.73 per cent by addition of dicalcium phosphate, graded amounts of calcium of 0.55, 0.71, 0.89, and 1.05 per cent, respectively, with corresponding Ca:P ratios of 0.7, 1.0, 1.2, and 1.4, respectively, induced increases of only very small magnitude in the rates of calcification of the animal body in the period in which calcification is normally most rapid. The animals on these different dietaries showed no significant differences in body length at a given age.

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THE EFFECT OF LIBERAL INTAKES OF CALCIUM OR CALCIUM AND PHOSPHORUS ON GROWTH AND BODY CALCIUM

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The earlier work of this laboratory showed a direct relation between the calcium content of the experimental animal (rat) and that of the dietary on which it had been nourished, up to 0.5 per cent of calcium in the dry food mixture (1, 2); and the experiments of Whitcher, Booher, and Sherman (3) suggest that still higher levels of calcium intake may be advantageous to calcium retention during the period of rapid growth. Meanwhile, in experiments continued throughout the life cycle, Sherman and Campbell (4) have shown that increasing the calcium intake from 0.2 per cent to 0.35 per cent of the dry food mixture results in more efficient use of food, somewhat more rapid growth, and greater adult vitality. In papers published since the experiments here described were performed, Cox and Imboden (5) suggest that, at different levels of intake of calcium and phosphorus, different ratios of calcium to phosphorus may be most advantageous for the nutritional support of gestation and lactation, and Shohl and Wolbach (6) emphasize the fact that discussion of ratio may be misleading unless adequacy of intake of each of these elements is sufficiently considered.

The plan of the present investigation was to increase systematically the calcium or calcium and phosphorus content of an already adequate diet, and seek to ascertain the level of calcium which (with a favorable Ca:P ratio) is most advantageous to the nutritional support of growth, of the normal developmental process of calcification, and of vitality and normal development through successive generations.

The effects of the experimental diets upon growth and body

calcium were studied especially in the second generations. Thus the determined differences here reported are the results not simply of an experimental period in the lives of the individuals analyzed but of a controlled comparison of intakes from the infancy of the parents, to and through the growth period of the animals analyzed. Thus the plan of experimentation was advanced an entire generation, and correspondingly a strikingly increased effect upon the calcium content of the young body was brought about.

EXPERIMENTAL

The starting points for the experiments here described were: (a) the mixture of 200 gm. of whole milk powder, 1000 gm. of ground whole wheat, and 20 gm. of sodium chloride, which constitutes the Diet A, Laboratory No. 16, of previous work in this Department; and (b) experimental animals from rat families which had thrived for 33 generations in this laboratory on this diet. This Diet 16, thus rigorously shown to be adequate, had a calcium content of 0.19 to 0.20 per cent, and a phosphorus content of 0.40 to 0.43 per cent, of the weight of the air-dry food mixture. This calcium intake may be judged, from results obtained by Campbell and coworkers (7) and from the general experience of this laboratory (8) to be near the minimum level for permanently adequate nutrition through successive generations. The transfer of animals raised on this Diet 16 to the new dietaries of the present investigation (Diets 168, 169, 268) involved no significant change in intake except in calcium or in calcium and phosphorus. These new dietaries were as follows:

By the addition of 13.8 gm. of calcium carbonate to the formula for Diet 16 above given, Diet 168 with 0.64 per cent¹ calcium (and a Ca:P ratio of 1.5) was prepared.

The further addition of 8.5 gm. of dibasic calcium phosphate to the dietary formula resulted in Diet 169, with 0.8 per cent calcium and 0.53 per cent phosphorus (the Ca:P ratio being thus approximately 1.5 as in Diet 168).

When, however, the formula for Diet 16 was enriched by the addition of 20 gm. of calcium carbonate alone, the resulting Diet

¹ This level of calcium intake was suggested by McCollum some years ago; but we find no record of its having been studied by methods comparable with those of the present investigation.

268 had (approximately) 0.8 per cent calcium (as in Diet 169) but only (approximately) 0.4 per cent phosphorus (as in Diet 16). Here the Ca:P ratio was widened to 2:1, which may be regarded either as due to increasing the calcium content of Diet 16 to a greater extent than in Diet 168, or as due to a lowering of the phosphorus content of Diet 169.

Table I summarizes the composition of these four diets in terms, (1) of the ingredients used in making them, and (2) of the results of chemical analyses of the completed mixtures reported in such round numbers as to avoid overemphasis on the natural variations in composition of the food materials used.

TABLE I
Composition of Diets

Diet No.....	16	168	169	268
Whole wheat, gm.....	1000	1000	1000	1000
“ milk powder, gm.....	200	200	200	200
NaCl, gm.....	20	20	20	20
CaCO ₃ , gm.....		13.8	13.8	20.0
CaHPO ₄ ·2H ₂ O, gm.....			8.5	
Ca, %.....	0.2	0.64	0.8	0.8
P, %.....	0.4	0.4	0.53	0.4
Ca:P ratio, about.....	0.5	1.5	1.5	2.0
Calories per 100 gm. diet.....	374	369	367	368
Protein, %.....	13.5	13.4	13.3	13.3

Experimental animals were grouped into matched lots at the age of 28 to 29 days. The phrase matched lots (or parallel lots), as used in this laboratory, means that each female in one lot is matched by a twin sister of essentially the same initial size, and each male by a twin brother of its own initial size, in the parallel lot (or in each of the parallel lots). The animals were allowed their respective food mixtures and distilled water *ad libitum*, so that each might eat according to its own energy requirement.

The original parallel lots with which each comparison was started have been continued on their experimental dietaries to test the respective values of the diets in the support both of growth and of the launching of a second generation. Young of this second generation have been analyzed for body calcium at fixed

ages to compare the values of the respective diets in facilitating the normal calcification of the body. Among animals destined for analysis the sexes were kept separate after the 56th day of age. Animals were killed by chloroforming at 30, 60, 90, and 180 days of age,² which covers the range of development from late infancy to early adulthood.

Before rigor mortis set in, the length of the body was measured.

In order to prepare the animal for analysis, the gastrointestinal tract was removed and its contents weighed and discarded. The live weight just before chloroforming minus the weight of the gastrointestinal contents gave the net weight of the rat. In some cases the whole animal was ashed in a silica dish at dull red heat. The ash was dissolved in hot dilute hydrochloric acid and the solution filtered and washed into a volumetric flask through a Whatman No. 44 filter paper. From this solution aliquot samples were withdrawn for calcium determination by the modified McCrudden method as used in this laboratory (1). In other cases the animal was obtained in dry form as described by Light, Smith, Smith, and Anderson (9). The whole rat was ground through a meat chopper into a tared aluminum pan. The chopper was cleaned with a brush and hot 50 per cent alcohol, so as to make as complete a recovery as possible. The ground material was dried at 60–65°. The final drying was done in a vacuum oven. The dry residue was ground until an apparently homogeneous mixture was obtained, from which representative samples could be taken for analysis. 2 gm. portions of the dry samples were weighed into small silica crucibles, burned in a muffle, and the percentage of ash determined. The ash was dissolved in hot dilute hydrochloric acid and the solution filtered into a beaker for calcium determination as before.

DISCUSSION

Growth in Body Weight—The animals on the original Diet 16 (0.2 per cent calcium) grew at a rate slightly below the average but within the normal zone. A 3-fold increase in the calcium content of the food (Diet 168) resulted in a definite increase in rate of gain in the body weight and also greater efficiency of growth

² When the stated day fell upon a Sunday, the animal was chloroformed the following morning.

whether calculated on the basis of energy or of protein intake. A further increase in the calcium and phosphorus content of the diet (Diet 169) did not in general cause further growth; while a calcium content of 0.8, with phosphorus of only 0.4 per cent (Diet

TABLE II
Average Body Weights at Different Ages and on Different Diets

	Diet No.	Ca in diet	P in diet	At 28 days		At 56 days		At 90 days		At 180 days	
				No. of cases	Weight	No. of cases	Weight	No. of cases	Weight	No. of cases	Weight
		per cent	per cent		gm.		gm.		gm.		gm.
Males	16	0.2	0.4	32	43	32	108	23	194	6	303
	168	0.64	0.4	26	46	26	122	15	220	5	330
	169	0.8	0.53	24	46	24	116	17	216	8	325
	268	0.8	0.4	18	46	18	110	12	198	6	302
Females	16	0.2	0.4	28	43	28	96	20	144	7	183
	168	0.64	0.4	20	45	20	120	15	162	6	196
	169	0.8	0.53	20	44	20	106	13	162	6	201
	268	0.8	0.4	17	42	17	99	12	155	6	184

TABLE III
Gains from 28th to 56th Day

Diet No.	Ca in diet	P in diet	Gain in body weight		Gain per 1000 Calories	Gain per gm. protein
			Males	Females		
	per cent	per cent	gm.	gm.	gm.	gm.
16	0.2	0.4	65 ± 2*	53 ± 2	71.8 ± 0.5	1.99 ± 0.06
168	0.64	0.4	76 ± 1	75 ± 2	74.5 ± 0.9	2.05 ± 0.03
169	0.8	0.53	70 ± 2	62 ± 1	77.6 ± 0.6	2.14 ± 0.01
268	0.8	0.4	64 ± 1	57 ± 1	66.3 ± 0.6	1.86 ± 0.02

* Probable error.

268), gave hardly better growth than that supported by the original Diet 16 (Tables II and III).

Growth in Body Length—The animals which were measured for body length when killed for analysis yielded the results shown in Table IV. As would be expected, there were hardly significant differences between the body lengths of the animals on the various

diets; but slightly greater body lengths resulted from the higher levels of calcium and phosphorus intake. Among the animals here measured, the males showed optimal growth in body length at a level of calcium intake of 0.64 per cent of the air-dry food mixture; and the females at 0.8 per cent. It is possible that the apparent difference is only accidental and that 0.64 to 0.80 may be taken as an optimal range regardless of sex.

Changes in Ash Content and Calcium Content of the Body—The extent of the calcification of the body was shown by the determina-

TABLE IV
Body Lengths at Different Ages

Diet No.	Ca in diet	P in diet	Ca, P, about	30 days	60 days	90 days	180 days
Males							
	per cent	per cent		cm.	cm.	cm.	cm.
16	0.2	0.4	0.5	12.6 ± 0.1*	16.6 ± 0.3	19.7 ± 0.2	22.4 ± 0.6
168	0.64	0.4	1.5	12.4 ± 0.1	17.5 ± 0.0	20.4 ± 0.1	23.4 ± 0.3
169	0.8	0.53	1.5	12.7 ± 0.1	18.1 ± 0.4	21.1 ± 0.2	23.1 ± 0.3
268	0.8	0.4	2.0	12.5 ± 0.2	17.3 ± 0.1	20.1 ± 0.2	23.2 ± 0.1
Females							
	per cent	per cent		cm.	cm.	cm.	cm.
16	0.2	0.4	0.5	12.5 ± 0.1	15.3 ± 0.3	17.0 ± 0.3	19.5 ± 0.1
168	0.64	0.4	1.5	12.1 ± 0.1	16.1 ± 0.2	18.3 ± 0.1	19.7 ± 0.1
169	0.8	0.53	1.5	12.0 ± 0.1	16.1 ± 0.2	19.0 ± 0.1	20.5 ± 0.1
268	0.8	0.4	2.0	12.0 ± 0.2	16.1 ± 0.1	18.6 ± 0.1	19.5 ± 0.1

* Probable error.

tion of calcium in the ash of the rat. Data for the body ash are shown in Table V.

The percentage of ash in the net body weight of animals from Diet 16 shows in these analyses an increase from about 3 per cent at 30 days to 3.4 per cent in males and 4.3 per cent in females at 180 days. At all ages here studied there were higher percentages of ash in the bodies of the animals reared on the diets of higher calcium content. A 4-fold increase in the calcium content of the diet (Diet 169) resulted in the highest percentages of body ash

here observed, varying with age (30 to 180 days) from 3.79 to 3.89 per cent for the males and 4.21 to 4.56 per cent for the females.

The calcium content of the body ash increased as the calcium in the diet was raised from 0.2 to 0.8 per cent of the dry food, and as the age of the rat increased. Hence, the use of the total ash content as an index of calcification or skeletal development is limited in accuracy by both the level of calcium in the diet and the age of the animal analyzed, so that actual determination of

TABLE V
Ash in Per Cent of Net Body Weight

diet No.	Ca in diet	P in diet	30 days	60 days	90 days	180 days
Males						
	per cent	per cent	per cent	per cent	per cent	per cent
16	0.2	0.4	3.05 \pm 0.06*	2.67 \pm 0.02	3.00 \pm 0.03	3.43 \pm 0.17
168	0.64	0.4	3.54 \pm 0.02	3.40 \pm 0.05	3.59 \pm 0.02	3.84 \pm 0.05
169	0.8	0.53	3.79 \pm 0.05	3.70 \pm 0.04	3.75 \pm 0.05	3.89 \pm 0.05
268	0.8	0.4	3.55 \pm 0.02	3.46 \pm 0.04	3.61 \pm 0.05	3.93 \pm 0.05
Females						
	per cent	per cent	per cent	per cent	per cent	per cent
16	0.2	0.4	2.98 \pm 0.03	2.85 \pm 0.03	3.36 \pm 0.03	4.32 \pm 0.07
168	0.64	0.4	3.63 \pm 0.02	3.64 \pm 0.03	3.99 \pm 0.03	4.49 \pm 0.05
169	0.8	0.53	4.21 \pm 0.01	3.99 \pm 0.07	4.26 \pm 0.07	4.56 \pm 0.05
268	0.8	0.4	3.89 \pm 0.18	3.65 \pm 0.05	4.00 \pm 0.03	4.57 \pm 0.05

* Probable error.

calcium is preferable when quantitatively exact findings are desired.

Table VI shows the weights and percentages of calcium in the bodies of the rats at different ages, and as influenced by the different dietaries here studied when each diet was continued into the second generation. Analyses of Diet 16 males at 30 days showed 0.71 per cent calcium, at 60 days in the middle of the fast growing period the gain in calcium has not kept pace with the gain in body weight, but from about 60 days the percentage of calcium again increases, and reaches 0.95 per cent of the body weight at 180 days. A 3-fold increase in the calcium content of the diet (Diet

168) resulted in the second generation males containing 0.95 per cent calcium at 30 days, a slightly lower percentage though a continuously increasing amount during the period of rapid growth, and 1.10 per cent at 180 days. At all ages here studied, there was a significant increase in the calcium content of the body in the animals on Diet 168 over those on Diet 16. A 4-fold increase in

TABLE VI
Influence of Family Diet on Body Calcium at Different Ages

Age	Family dietary			Male rats				Female rats			
	Diet No.	Ca in diet	P in diet	No. of cases	Net weight	Ca in body	Ca in body	No. of cases	Net weight	Ca in body	Ca in body
	days	per cent	per cent		gm.	gm.	per cent		gm.	gm.	per cent
30	16	0.2	0.4	10	45	0.29	0.71 ± 0.01*	12	43	0.30	0.72 ± 0.01*
	168	0.64	0.4	16	42	0.39	0.95 ± 0.01	8	43	0.41	0.96 ± 0.01
	169	0.8	0.53	12	41	0.42	1.03 ± 0.01	9	39	0.41	1.10 ± 0.01
	268	0.8	0.4	6	44	0.41	0.95 ± 0.01	5	42	0.40	0.96 ± 0.02
60	16	0.2	0.4	9	112	0.73	0.65 ± 0.01	8	92	0.64	0.69 ± 0.01
	168	0.64	0.4	11	115	1.19	0.94 ± 0.01	5	108	1.11	1.03 ± 0.01
	169	0.8	0.53	7	107	1.24	1.02 ± 0.02	7	95	1.05	1.11 ± 0.01
	268	0.8	0.4	6	112	1.07	0.96 ± 0.02	5	100	1.00	1.00 ± 0.01
90	16	0.2	0.4	11	186	1.38	0.74 ± 0.01	7	129	1.12	0.86 ± 0.01
	168	0.64	0.4	10	206	2.05	0.99 ± 0.01	9	150	1.74	1.19 ± 0.01
	169	0.8	0.53	9	209	2.18	1.05 ± 0.01	7	155	1.91	1.23 ± 0.02
	268	0.8	0.4	6	188	1.93	1.02 ± 0.01	6	151	1.74	1.16 ± 0.01
120	16	0.2	0.4	5	224	2.00	0.89 ± 0.01	6	157	1.63	1.03 ± 0.03
180	16	0.2	0.4	6	289	2.75	0.95 ± 0.01	7	187	2.38	1.24 ± 0.03
	168	0.64	0.4	5	316	3.50	1.10 ± 0.01	6	183	2.45	1.33 ± 0.02
	169	0.8	0.53	8	297	3.52	1.14 ± 0.01	6	191	2.64	1.38 ± 0.02
	268	0.8	0.4	6	284	3.20	1.14 ± 0.01	6	173	2.38	1.38 ± 0.01

* Probable error.

the calcium content of the diet with a moderate increase in the phosphorus content (Diet 169, 0.8 per cent calcium, 0.53 per cent phosphorus) gave young male rats of 1.03 per cent calcium at 30 days of age, which increased to 1.14 per cent calcium at 180 days. There was a definite increase in the calcium content of these rats over those of Diet 168 (0.64 per cent calcium) during growth; but hardly a significant difference in the young adult.

Diet 268 (0.8 per cent calcium but only 0.4 per cent phosphorus) did not give as great an early calcium retention as that which accompanied a simultaneous increase in the phosphorus intake (Diet 169), though here again the percentage of calcium in the adult animals was about the same. Yet because they remained smaller, the animals on Diet 268, even at adult age, had not stored as much calcium in gm. as had those on Diet 169.

Thus the effect of increasing the calcium to 0.64 to 0.8 per cent in the otherwise favorably constituted diet of the rat has been shown to be a greater growth, a more efficient use of the food in growth, and an enhancement of the normal process of calcification in the growing body. The animals analyzed were the offspring of parents which had been from infancy on the same respective diets, so that the influence of the higher calcium or calcium and phosphorus content of each new diet was given fuller opportunity for manifestation than in feeding experiments of the more usual type. Such observations at later stages of the life cycle as have yet been possible have indicated also a greater adult vitality on the diets of higher calcium content.

SUMMARY

Increase in the calcium or calcium and phosphorus content of an already adequate diet resulted in more rapid and more efficient growth, with greater economy in the use of the food. The rate of normal calcification as shown in young of the second generation was also distinctly increased.

On dietaries known to be adequate in other respects, the full advantage to growth and apparent general health and vitality seemed to have been attained at a level of intake of 0.64 per cent of calcium in the air-dry food mixture; but further increase to 0.8 per cent calcium may still further increase the rate of calcification.

30 day-old rats from families on adequate diet of nearly minimal calcium content (0.2 per cent) had about 0.6 the percentage of body calcium found in normal adults; those from families whose food contained 0.64 per cent calcium (Ca:P ratio of 1.5) had about 0.8; and those from families whose food contained 0.8 per cent calcium (Ca:P ratio of 1.5) had about 0.9 of the same normal adult percentage.

In the experiments here reported, increasing the calcium content of an adequate diet to 0.8 per cent of the weight of dry food mixture has not revealed any unfavorable result to throw doubt upon the advantage of the increased rate of growth and normal calcification.

While the work here described has been separately supported, the fact that it has profited by coordination with the experiments which are being carried on in this laboratory through the cooperation of the Carnegie Corporation of New York and the Carnegie Institution of Washington, is gratefully acknowledged.

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SOME ASPECTS OF PROTEIN INTAKE IN RELATION TO GROWTH AND RATE OF CALCIFICATION

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In accordance with the view emphasized by McCollum and Simmonds (1) that experiments extending over significant parts of the life cycle tend to yield higher and more valid impressions of the level of protein intake needed for the best permanent results than do the relatively much shorter studies of nitrogen balance, it suffices here to mention those previous investigations in which test animals under laboratory control have been fed graded proportions of protein (with or without simultaneous study of the mineral intake) throughout a considerable percentage of the period of growth, or longer. In each of the investigations here discussed the rat has been chosen as the experimental animal, largely because the chemistry of its metabolism so closely resembles that of the human (2).

Steenbock, Kent, and Gross (3) found that barley alone was unable to meet the nutritional needs of growth and that a generous addition of casein alone was of little if any benefit, or even appeared deleterious; but when the barley diet was enriched both with casein and a suitable salt mixture the nutritional results were better than those obtained by the addition of the salt mixture alone.

Osborne and Mendel (4) reported that as they progressively increased the protein content of an adequate diet from 18 per cent to 35 per cent there was a corresponding increase in the rate of growth. Smith and Anderson (5) found excellent growth in animals fed a diet containing 22 per cent protein. Slonaker (6) in experiments covering growth, reproduction, and length of life, obtained best results with a protein level of 14 per cent of the

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weight of air-dry food. There appeared no significant change in nutritional well being when this level was either lowered to 10 per cent or increased to 18 per cent; but at 22 per cent the results on the whole were less favorable.

Plan of Present Work

The experiments here reported were designed in order to study the effects of protein enrichments of diets known to be adequate to all the nutritional needs of the species, including tests at different levels of calcium intake within the normal range, previous work in this laboratory (7) having shown the possibility that an already adequate diet may still be capable of improvement by a more scientific adjustment of the quantitative proportions of its constituents.

The starting point for these various enrichments was Diet 16 of this laboratory (also designated in some of the previous papers as Diet A). It consists of five-sixths ground whole wheat and one-sixth dried whole milk with sodium chloride added in an amount equivalent to 2 per cent of the weight of the wheat. From several analyses, it has been found to average 0.19 to 0.2 per cent calcium, 0.44 per cent phosphorus, and 14.0 per cent protein in the air-dry food mixture.

In the series of experiments here chiefly described, this diet was enriched in its protein content with or without a parallel enrichment in calcium alone or both calcium and phosphorus. In supplementary experiments the protein content was again increased to approximately 25 and 40 per cent respectively, while essentially the same calcium and phosphorus contents were maintained as in the first series. In some of the latter experiments the diet of higher protein content was also enriched in its vitamin values, in order to coordinate the present work with that published by McCay, Crowell, and Maynard (8) while our chief series of experiments was in progress.

EXPERIMENTAL AND DISCUSSION

Young, healthy, albino rats, which had been reared to 28 to 30 days of age by mothers on Diet 16, were grouped into three matched lots. Each lot, consisting of two males and three females, was housed in a 12 × 15 inch galvanized iron cage. Food and

distilled water were available *ad libitum*. As the sexes grew up together, mating occurred as soon as the animals were sufficiently mature. Strictly parallel lots were placed on each of the three diets described in Table I. As more than one generation may be required for the full effect of a difference of diets, parallel lots of offspring of the original matched lots in the second and third generations were formed, these lots consisting either of two males and three females, or in a few cases of two males and two females.

When the formation of lots of successive generations was completed, the remaining offspring of the females of the original matched lots, as well as those obtained from the females of suc-

TABLE I
Composition of Diets Used in Chief Series of Experiments

Constituents	Diet 170		Diet 171		Diet 172	
	Per cent by weight	Weight	Per cent by weight	Weight	Per cent by weight	Weight
		gm.		gm.		gm.
Whole wheat.....	78.07	1000.0	77.18	1000.0	76.67	1000.0
“ milk.....	15.61	200.0	15.42	200.0	15.33	200.0
NaCl.....	1.56	20.0	1.54	20.0	1.53	20.0
Casein.....	4.76	61.0	4.78	62.0	4.75	62.0
CaCO ₃			1.06	13.8	1.06	13.8
CaHPO ₄ ·2H ₂ O....					0.65	8.5
Ca.....	0.18-0.2		0.61-0.64		0.77-0.8	
P.....	0.42-0.44		0.42-0.44		0.53	
Protein.....	18.7-18.8		18.7-18.8		18.7-18.8	

cessive generations, were either reared on the diets of their mothers to various ages, when their bodies were analyzed for calcium content, or placed on diets of higher protein content as described below.

Growth and Calcification of Animals of Main Series

Growth—All animals grew normally and presented the physical appearance of good health and development. Table II gives the average growth in gm. from the 28th to 56th day of both male and female rats on each diet. Group 1, Table II, represents the average growth of the animals in the lot cages; Group 2, the average of the animals reared for analysis (*i.e.*, bachelor and virgin rats);

and Group 3, the combined average of Groups 1 and 2. Table III compares the results here found at different calcium levels in diets of 18.7 to 18.8 per cent protein with the corresponding data of Toepfer and Sherman's experiments with diets containing 14 per cent of protein.

TABLE II
Average Gain in Weight of Animals, 28th to 56th Day

	Group No.	Diet 170		Diet 171		Diet 172	
		No. of cases	Average gain	No. of cases	Average gain	No. of cases	Average gain
			gm.		gm.		gm.
Males	1	8	83±2*	10	88±6*	10	98±2*
	2	22	77±2	19	89±3	19	87±2
	3 (1 + 2)	30	79±2	29	88±3	29	91±1
Females	1	11	58±2	14	63±4	15	76±1
	2	22	62±1	24	59±1	20	66±2
	3 (1 + 2)	33	60±1	38	60±2	35	70±1

* ± average deviation of the mean.

TABLE III
Average Gains between 28th and 56th Days of Age, in Rats on Diets Differing in Protein and Calcium Contents

Calcium in diet	Protein in diet	Males		Females	
		No. of cases	Gain in weight	No. of cases	Gain in weight
<i>per cent</i>	<i>per cent</i>		gm.		gm.
0.18-0.2	14.0	32	65	28	53
	18.7	30	79	33	60
0.61-0.64	14.0	26	76	20	75
	18.8	29	88	38	60
0.77-0.81	14.0	24	70	20	62
	18.8	29	91	35	70

In five of these six comparisons, the enrichment of the dietary in protein content resulted in more rapid growth.

Diets 170, 171, 172 have also been compared as to the average weight of the animals of both sexes on these diets at various ages up to 180 days. The results of this comparison are given in Table IV. As representative animals were taken for analysis at

stated ages, the numbers of cases for which weight data were obtained necessarily diminish with increasing age. The averages given are for both mated and unmated males; but for females only the data of those unmated are here used in order to avoid the fluctuations incident to pregnancy, parturition, and lactation.

The 3- to 4-fold more liberal intake of calcium has induced a favorable response in the growth of the male animals, not only during the period uncomplicated by breeding but throughout the entire period of rapid growth. Somewhat different results were

TABLE IV

Average Weights of Rats on Diets 170, 171, and 172, Compared at Various Ages

	Age	Diet 170		Diet 171		Diet 172	
		No. of cases	Average weight	No. of cases	Average weight	No. of cases	Average weight
	<i>days</i>		<i>gm.</i>		<i>gm.</i>		<i>gm.</i>
Males	30	35	52±1*	33	56±1*	33	50±1*
	60	28	138±2	29	151±3	28	148±2
	90	23	204±3	24	222±4	22	227±3
	120	19	247±5	19	270±5	15	270±3
	150	16	279±5	16	303±7	11	302±2
	180	13	303±5	13	327±8	9	330±2
Females	30	29	50±1	26	52±1	25	50±1
	60	22	115±1	22	110±2	20	112±2
	90	16	151±1	17	149±3	14	148±4
	120	11	171±2	11	178±2	9	166±4
	150	7	190±2	8	189±3	8	192±2
	180	5	196±5	4	200±5	5	196±4

* ± average deviation of the mean.

obtained with the females. Although the average results were not established with as much precision as in the case of the males, owing to the smaller number of cases at each age, it would appear from Table IV that the average size of the females at the various ages was not affected by a more liberal intake of calcium, as was the case with the male animals on the same level of protein feeding (18.8 per cent).

Table V compares the average weights at the ages of 30, 60, 90, and 180 days, obtained in this laboratory on diets of differing

protein and calcium contents, as described in this and the preceding paper.

For the males, the data of Table V show that enriching Diet 16 (already adequate but of low calcium content) in protein alone has resulted in a somewhat more rapid growth, although the average adult weight attained at 180 days was approximately the same whether the diet contained 14 or 18.8 per cent of protein.

TABLE V

Comparison of Average Weights of Animals on Diets Differing in Protein and Calcium Contents

	Diet No.	Age							
		30 days		60 days		90 days		180 days	
		No. of cases	Average weight	No. of cases	Average weight	No. of cases	Average weight	No. of cases	Average weight
Males			gm		gm.		gm.		gm.
	16	32	38	32	91	23	194	6	303
	170	35	52	28	138	23	204	13	303
	168	48	48	31	134	15	220	5	330
	171	33	56	29	151	24	222	13	327
	169	30	48	22	126	17	216	8	325
	172	33	50	28	148	22	227	9	330
Females	16	32	36	32	77	20	144	7	183
	170	29	50	22	115	16	151	5	196
	168	36	48	27	122	15	162	6	196
	171	26	52	22	110	17	149	4	200
	169	25	46	20	114	13	162	6	201
	172	25	50	20	112	14	148	5	196

Increasing the protein content of a diet containing a 3-fold or a 4-fold enrichment in calcium content over that of Diet 16 (Diets 168 and 169, respectively) has resulted in a somewhat more rapid growth in the males up to 90 days of age, when the average weight of the animals on the two diets becomes approximately the same. Thus these data as a whole show that the growth of the males was more rapid with 18.8 per cent protein than with 14.0 per cent pro-

tein in the diet, whether its calcium content was 0.2, 0.6, or 0.8 per cent of the dry weight of the food mixture.

With the female animals, the result of increasing the protein content of the various diets is not as clearly defined as in the case

TABLE VI

Average Calcium Content of Rats at Different Ages on Diets Containing 18.8 Per Cent of Protein and from 0.18 to 0.77 Per Cent of Calcium

	Age	Diet No.	No. of cases	Ca in diet	Average body weight	Average net body weight	Total Ca in body	
				per cent	gm.	gm.	gm.	per cent
Males	30	170	4	0.18	54	48	0.27 \pm 0.03*	0.60 \pm 0.02*
		171	4	0.61	59	53	0.46 \pm 0.01	0.88 \pm 0.02
		172	3	0.77	58	52	0.47 \pm 0.04	0.90 \pm 0.02
Females	30	170	4	0.18	49	44	0.30 \pm 0.02	0.65 \pm 0.005
		171	5	0.61	49	44	0.41 \pm 0.01	0.94 \pm 0.02
		172	4	0.77	54	47	0.46 \pm 0.01	0.98 \pm 0.01
Males	60	170	4	0.18	141	122	0.83 \pm 0.04	0.67 \pm 0.02
		171	4	0.61	162	147	1.35 \pm 0.02	0.91 \pm 0.01
		172	5	0.77	149	135	1.35 \pm 0.07	0.99 \pm 0.03
Females	60	170	4	0.18	120	108	0.68 \pm 0.01	0.74 \pm 0.02
		171	4	0.61	113	101	1.09 \pm 0.02	1.07 \pm 0.01
		172	4	0.77	115	102	1.10 \pm 0.04	1.09 \pm 0.02
Males	90	170	3	0.18	212	199	1.46 \pm 0.10	0.72 \pm 0.04
		171	4	0.61	219	203	2.03 \pm 0.08	1.00 \pm 0.02
		172	5	0.77	218	205	2.11 \pm 0.07	1.02 \pm 0.01
Females	90	170	4	0.18	153	142	1.29 \pm 0.03	0.90 \pm 0.006
		171	5	0.61	137	124	1.53 \pm 0.08	1.23 \pm 0.02
		172	5	0.77	152	138	1.73 \pm 0.05	1.25 \pm 0.01
Males	180	170	6	0.18	292	277	2.56 \pm 0.19	0.92 \pm 0.005
		171	5	0.61	313	298	3.09 \pm 0.11	1.03 \pm 0.008
		172	4	0.77	325	309	3.39 \pm 0.07	1.08 \pm 0.001
Females	180	170	4	0.18	200	188	1.97 \pm 0.24	1.10 \pm 0.01
		171	4	0.61	194	183	2.24 \pm 0.10	1.22 \pm 0.01
		172	4	0.77	194	183	2.28 \pm 0.03	1.29 \pm 0.01

* \pm average deviation of the mean.

of the males; but there appears a significant relation between growth and calcification.

Calcium Content of Animals—As has been previously mentioned, 28 to 29 day-old offspring of the animals on Diets 170 to 172 were

reared on the diets of their mothers to definite ages and their bodies analyzed for calcium at 30, 60, 90, and 180 days of age.

These animals were kept in individual cages with food and distilled water available *ad libitum*. All of them grew normally and were apparently in good health. The animals were killed with chloroform and brushed to remove any food or other foreign particles from the fur. The gastrointestinal tract was dissected out, the contents removed, and the weight of the contents subtracted from the live weight of the rat to give net weight, which was used as a basis in all calculations. The bodies were prepared for analysis and calcium determined as previously described (9-11). The results are summarized in Table VI.

These analyses show that in the second generation of families on diets of liberal protein content (18.8 per cent of the air-dry food mixture) the diet having a calcium content of 0.6 or 0.8 per cent of the dry matter had enabled the body to attain a calcium content of 0.88 to 0.90 per cent in the males and 0.94 to 0.98 per cent in the females at 30 days of age, as compared with 0.60 per cent in the males and 0.65 per cent in the females at the same age on the diet having a calcium content of 0.2 per cent, although this latter meets the minimal needs of normal nutrition. This difference is essentially the same as that found by Toepfer and Sherman (11) in corresponding animals on diets of lower protein content (14 per cent). The rate at which the percentage of calcium in the body approaches that of the normally developed young adult is also very similar for the two groups which received diets otherwise alike but containing approximately 14 per cent and 18.8 per cent of protein respectively.

Among the animals on the diet of approximately minimal adequate (0.2 per cent) calcium content, enrichment of the protein content of the diet from 14 per cent to 18.8 per cent resulted in a somewhat increased gain of body calcium, especially in the period of most rapid growth (around the ages of 60 to 90 days), which difference had practically disappeared at 180 days.

Among those on diets of liberal (0.6 to 0.8 per cent) calcium content, the effect of similar enrichment of the dietary with protein was apparently more complex. In the cases in which the animals responded to the extra protein feeding by increasing their rate of growth, there appears to have been also an increase in the rate of

calcification, while in the cases in which the extra protein did not increase the growth rate, the calcification was no more rapid or was even somewhat less rapid than on the lower level of protein feeding.

Experiments with Diets of Higher Protein Contents

As above noted, growth was sometimes but not always more rapid with protein at 18.8 per cent than at 14 per cent in our experiments. It is also of interest in this connection that in the experiments of McCay, Crowell, and Maynard (8) a diet which, along with liberal proportions of yeast and of cod liver oil, contained 40 per cent of protein in the dry food mixture resulted in growth which was judged to be too rapid for best results, inasmuch as restriction of growth by limiting the allowance of this rich diet was followed by increased longevity of the males—though not of the females. With a view to exploring the area between the Cornell experiments and those described above, we have begun experimentation with diets containing 25 per cent and 40 per cent of protein.

For the experiments with dietaries enriched with protein to 25 and to 40 per cent without other change (Diets 673 to 676), young animals from families on Diets 171 and 172 were used. Experiments with diets of 40 per cent protein content plus vitamin enrichment (Diets 850 to 852) were started with matched lots of young animals from other families belonging to the same strain.

The compositions of the diets are summarized in Table VII; and the average growth of the rats on each diet from their 28th to 56th days of age is shown in Table VIII.

At the lowest level of calcium intake here studied (0.18 to 0.22 per cent calcium in the dry food mixture), the increase of the protein content of the diet from 18.7 per cent to 25 per cent of the dry weight of the food and also the further increase to 40 per cent of protein with or without vitamin enrichment have here resulted in more rapid growth of the males but not of the females. This is suggestive, in view of the fact that in the experiments of McCay, Crowell, and Maynard with diets of high protein and vitamin content the males but not the females seemed (when the food was furnished *ad libitum*) to grow more rapidly than was consistent with subsequent longevity.

TABLE VII
Composition of Diets of 25 and 40 Per Cent Protein Content

Diet No.	673	674	675	676	850	851	852
Whole wheat, gm.	1000.0	1000.0	1000.0	1000.0	472.0	462.0	455.0
Dried whole milk, gm.	200.0	200.0	200.0	200.0	94.0	94.0	94.0
NaCl, gm.	20.0	20.0	20.0	20.0	9.0	9.0	9.0
Casein, gm.	198.0	557.0	201.0	562.0	325.0	325.0	325.0
CaCO ₃ , gm.	17.6	25.1	18.7	26.4		10.0	10.0
CaHPO ₄ ·2H ₂ O, gm.			8.5	8.5			7.0
Cod liver oil, gm.					50.0	50.0	50.0
Dried yeast, gm.					50.0	50.0	50.0
Ca, %	0.65	0.68	0.81	0.82	0.22	0.59	0.79
P, %	0.46	0.48	0.56	0.57	0.41	0.42	0.57
Protein, %	25	40	25	40	40.1	39.9	39.8

TABLE VIII
Comparison of Average Gain in Weight of Animals from 28th to 56th Day on Diets Differing in Composition

Diet No.	Ca in diet	P in diet	Protein in diet	Males		Females	
				No. of cases	Average gain in weight	No. of cases	Average gain in weight
	per cent	per cent	per cent		gm.		gm.
168	0.64	0.46	14.0	26	76±<1*	20	75±2*
171	0.61	0.43	18.8	29	88±3	38	60±2
673	0.65	0.46	25	4	121±4	9	82±2
674	0.68	0.48	40	4	91±4	7	79±2
851†	0.59	0.42	39.9	11	97±3	4	64±2
169	0.81	0.53	14.0	24	70±1	20	62±1
172	0.77	0.53	18.8	29	91±1	35	70±1
675	0.81	0.56	25	7	104±2	14	83±1
676	0.82	0.57	40	9	109±3	10	85±2
852†	0.79	0.57	39.8	6	107±6	4	68±<1
16	0.19	0.44	14.0	32	65±1	28	53±1
170	0.18	0.43	18.7	30	79±2	33	60±1
850†	0.22	0.41	40.1	9	111±5	5	56±1

* ± average deviation of the mean.

† Diets enriched in their vitamin values.

In those of our experiments in which the level of calcium supply was liberal (0.6 to 0.8 per cent of calcium in the dry food mixture), increase of protein from 18.7 to 25 per cent of the dry weight of

food increased the growth of both males and females; while a further increase of protein to 40 per cent, with or without simultaneous enrichment in vitamin values, has not resulted in more rapid growth than that shown by the animals receiving the diets of 25 per cent protein content.

Studies of the relations of these further enrichments to the rate of calcification are in progress.

• SUMMARY

Starting with a diet containing 0.2 per cent of calcium in the dry matter (a nearly minimal adequate level), we found that an increase in the protein content of this diet from 14 per cent to 18.8 per cent resulted in a more rapid growth, measured by gain in weight during the period from the 28th to the 56th day of age in the rat, and also an increased rate of calcification.

With diets of liberal normal calcium content (0.64 to 0.8 per cent), increase of protein content from 14 to 18.8 per cent sometimes did and sometimes did not increase the rate of growth. When the rate of growth was thus increased, the rate of normal calcification was also increased.

Increasing the protein content of these diets of more liberal calcium intake to 25 per cent of protein in the air-dry food mixture induced a further increase of growth in both male and female rats during the period from the 28th to the 56th day of age. A further increase in the protein content of these diets to 40 per cent of the air-dry food mixture with or without a simultaneous enrichment in vitamin values did not increase the rate of growth of either males or females over that obtained on a diet containing 25 per cent protein.

While the work here described has been separately supported, the fact that it has profited by coordination with the experiments which are being carried on in this laboratory through the cooperation of the Carnegie Corporation of New York and the Carnegie Institution of Washington, is gratefully acknowledged.

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A NEW ESSENTIAL DIETARY FACTOR*

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We have shown in our previous work (1, 2) that flavins are inactive in the cure or prevention of pellagra-like symptoms in the chick and black tongue in dogs, and that a fraction prepared from liver extract from which the flavin had been removed by adsorption on fullers' earth was highly active in the prevention of both these syndromes. Recently Dann (3), using the technique of Ruffin and Smith (4), tested the efficacy of lactoflavin in the treatment of human pellagra. Of the three pellagrins which he studied, none responded to lactoflavin, although two of them, when subsequently given liver extract, showed remission of symptoms. It is evident, therefore, that flavins are not the human pellagra-preventive factor.

In another paper by Birch, György, and Harris (5), which appeared after the work reported in this paper was initiated, the following conclusion is made, "The human pellagra-preventing ('P.-P.') factor is different both from vitamin B₆ (hitherto called the 'rat pellagra' factor) and from lactoflavin, two known components of the vitamin B₂ complex." These workers also state that the so called chicken pellagra of Elvehjem and Koehn appears to be distinct from vitamin B₆ deficiency, and its relation to human pellagra and black tongue is undecided. The relation of the factor remaining in the liver extract preparation after removal of flavins

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to black tongue is no longer undecided because we (2) have shown the fraction to be highly active in the cure of black tongue.

Since most of the work on vitamin B₂ has been carried out with rats as the experimental animal, we were interested in using rats for assaying the potency of the factor remaining in the fullers' earth filtrate from liver extract. Since this factor is probably identical with or closely related to the human pellagra-preventive factor, we will continue to designate it vitamin B₂ until the nomenclature is settled by the proper authorities.

Goldberger and Lillie (6) were the first to produce pellagra-like symptoms in rats on diets in which the vitamin B complex was supplied as an 85 per cent alcohol extract of corn-meal. They considered the dermatitis in rats to be analogous to human pellagra. The greatest difficulty has been encountered in incorporating vitamin B₁ and other essential factors into synthetic rations without adding appreciable amounts of the vitamin being studied. Chick and Roscoe (7) used a vitamin B₁ concentrate prepared by Kinnersley and Peters; Bourquin and Sherman (8) an alcoholic extract of wheat; and Hogan and Richardson (9) irradiated yeast. The potency of the material assayed was generally based upon rate of growth in unit time. Several workers, Chick and Roscoe (10), Salmon, Hayes, and Guerrant (11), and Sure, Smith, and Kik (12), have reported a lack of correlation between the failure of growth and the incidence of pellagra, but in spite of these observations growth has continued to be used as the criterion of potency of vitamin B₂ concentrates.

Diets compounded according to the Bourquin-Sherman formula have been employed most extensively in vitamin B₂ studies. György, Kuhn, and Wagner-Jauregg (13), using this type of diet, found 5 micrograms of lactoflavin to stimulate growth in rats if the ration were supplemented with a further fraction obtained from yeast. Chick and Copping (14) had produced evidence that yeast contained in addition to vitamins B₁ and B₂ a dietary factor designated as factor Y. Later György (15), Chick, Copping, and Edgar (16), and Harris (17) demonstrated that the "complimentary factor" and not the lactoflavin is the one which protects rats against the so called pellagra-like dermatitis. György (15) named the complimentary factor vitamin B₆ and Copping (18) in a very recent paper fully confirms the conclusions of György

and shows that flavin prevents an affection of the skin of rats in which the hair is shed without swelling or inflammation and vitamin B₆ prevents symmetrical floriid dermatitis.

Thus a synthetic diet in which an alcoholic extract of grains is used will vary depending upon the amount of flavin, vitamin B₆, and other factors which this extract carries. This variation has been discussed by both Birch, György, and Harris (5) and by Copping (18).

In our work we tried to devise a ration as synthetic as possible which contained all of the vitamin B complex except vitamin B₂.

TABLE I
Constituents of the Various Diets

Ration	Starch	Dextrin	Sucrose	Casein	Salts I	Cod liver oil*	Peanut oil	Butter fat	Crisco	Cottonseed oil	White corn	Vitamin B ₁ μgm. per kg
K ₁	66			20	4		5	5				200
K ₂	66			20	4	2	8					200
K ₄			66	20	4		5	5				200
K ₅			64	18	4	2		5	7			200
K ₈			64	18	4	2				12		400
K ₉			67	18	4	2			10			200
K ₁₀			64	18	4	2		5	7			200
K ₁₁		49		18	4	2		5		9	12	300
K ₁₂		58		18	4	2		5			12	200

* Rats not receiving cod liver oil were irradiated twice weekly.

However, completely negative results were obtained with a ration supplying ample amounts of vitamins B₁, B₂, B₄, B₆, and flavins. Thus our ration was deficient in an additional unrecognized factor which we wish to describe in this paper.

EXPERIMENTAL

The casein for our synthetic rations was prepared from skim milk by precipitation with HCl, decanting, washing, dissolving in ammonia, and repeating the process three times. The butter fat was freed from curd by melting and filtering. Salts I described by Kline, Bird, Elvehjem, and Hart (19) were used.

In the first trials, several modifications involving carbohydrate, fat, and vitamin supplements were tried (Rations K₁, K₃, K₄, K₅, K₈, and K₉, Table I). Typical growth curves of rats placed on several of these rations are shown in Chart I. It is readily seen that rats on the vitamin B-free rations plus vitamin B₁ completely failed to grow. They also failed to show any symptoms which might aid in determining the specific deficiencies. When these rations were supplemented with 2 per cent of ether extract of wheat germ, which is known to contain the factor described by Hogan and Richardson (20), and which has a curative action on

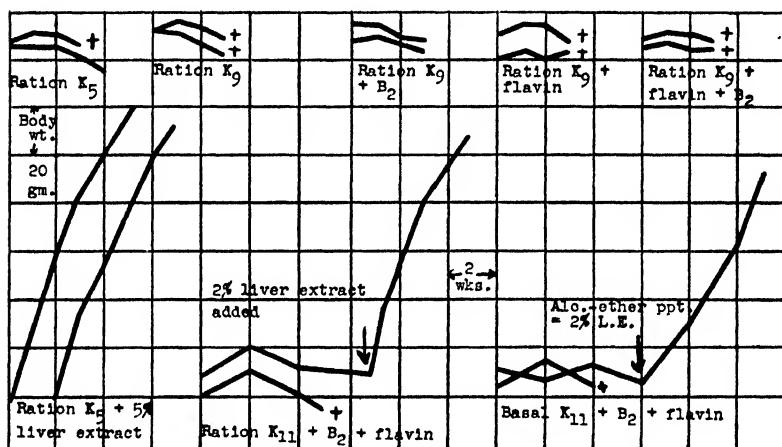


CHART I. Growth curves for rats on the basal ration and the basal ration supplemented with vitamin B₂, flavin, crude liver extract, and alcohol-ether precipitate fraction.

vitamin B₆-deficient animals (21), no improvement was noted. However, when 5 per cent liver extract was added to these rations, excellent growth resulted. Typical growth curves are shown in Chart I. Apparently 5 per cent liver extract supplied all of the missing factors of the vitamin B complex.

Our next step seemed quite obvious. Rats from the same litter were divided into three groups at the age of weaning. One group received the basal Ration K₉ plus flavin, one group basal Ration K₉ plus vitamin B₂, and one the basal ration plus both flavin and vitamin B₂. The flavin and vitamin B₂ fractions were prepared

according to the method of Koehn and Elvehjem (2). Typical results are shown in Chart I. Complete failure of growth resulted in all cases. When basal Rations K_{10} and K_{11} were used with the same supplements, similar results were obtained. Since Ration K_{11} contained 12 per cent corn, the deficiency could not be one due to lack of vitamin B_6 . Increasing the levels of vitamin B_2 and flavins had no effect. However, when basal Ration K_{11} was supplemented with as little as 2 per cent liver extract, the rats developed normally (Chart I).

It is very evident that the addition of either flavin or vitamin B_2 independently or together had no effect on growth. Thus the basal ration was deficient in constituents of the vitamin B complex other than B_1 , B_2 , B_6 , and flavins. Since liver extract alone produced excellent growth, while the flavin and vitamin B_2 concentrates gave completely negative results, the other factor or factors must be present in liver extract. We turned our attention, therefore, to the various fractions from the liver extract, which might carry additional factors. The two fractions in the preparation of the flavin and vitamin B_2 concentrates from liver extract, previously described (2), which were discarded and which might be active, were the alcohol-ether precipitate, and the fullers' earth after elution of the flavins.

When the alcohol-ether precipitate equivalent to 2 per cent liver extract was added to basal Ration K_{11} supplemented with flavin and vitamin B_2 , phenomenal growth response was observed (Chart I). Thus a factor distinct from vitamins B_1 , B_2 , B_6 , and flavins necessary for the growth of rats was contained in this fraction.

Rats placed on Ration K_{10} supplemented with the alcohol-ether precipitate, flavins, and vitamin B_2 for a long period of time exhibited symptoms of paralysis typical of a vitamin B_4 deficiency. This condition could be prevented by the addition of 5 per cent of ground peanuts or 12 per cent of ground white corn, supplements shown in this laboratory to be good sources of vitamin B_4 . The best results were obtained with Ration K_{12} in which white corn furnished the vitamin B_4 , as well as vitamin B_6 , and which contained a lower percentage of fat.

Since we now had a complete ration, we attempted to produce pellagra by supplementing basal Ration K_{12} plus the precipitate

factor with flavins, and to produce flavin deficiency by supplementing with vitamin B₂. However, basal Ration K₁₂ plus the precipitate factor alone produced the same growth as that obtained when vitamin B₂ and flavin were also supplied (Chart II). Evidently the fraction containing the precipitate factor was contaminated with flavin and vitamin B₂. Therefore, we concentrated our efforts on purifying the precipitate.

Assay—For studying the new factor, we have used basal Ration K₁₂ and have relied upon growth records as an indication of activity. Although this factor has a profound effect on the develop-

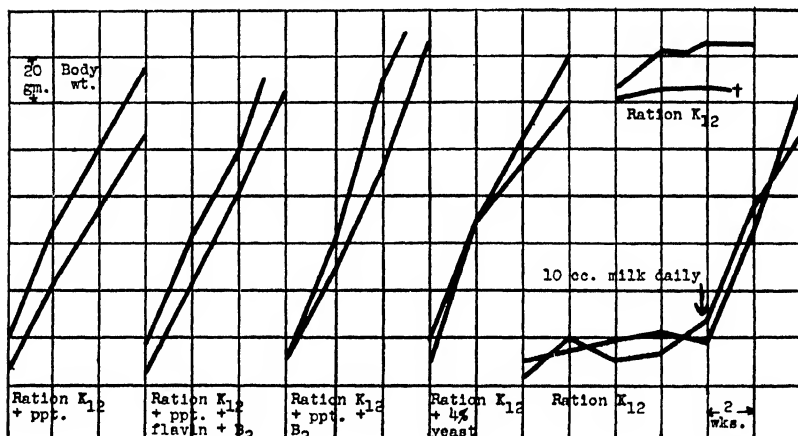


CHART II. Growth curves for rats on basal Ration K₁₂ and the basal ration plus various supplements containing the alcohol-ether precipitate factor.

ment of the rat, no definite pathological symptoms have been observed in rats suffering from this deficiency other than lack of growth and general emaciation.

Distribution and Identity—Many workers have reported that rats fed a diet devoid of vitamins of the B complex grew normally, if the ration was supplemented with yeast or an aqueous extract of yeast. We were interested therefore in determining the potency of yeast in respect to our precipitate factor. We found that dried bakers' yeast was a good source of this factor, producing excellent growth at a 4 per cent level (Chart II). Whole milk was found to produce an immediate growth response when 10 cc. were fed daily (Chart II).

Since the general behavior of the factor indicated that it might be proteinaceous in nature, products of this type were assayed. Arginine, cystine, and choline were completely inactive, as was blood fibrin fed at 2, 5, and 10 per cent levels. Crude casein, brain, and lung tissue were found to be inactive. Liver residue remaining after the liver extract was prepared showed practically no potency even at a 10 per cent level. Increasing the levels of vitamin B₁, B₂, and flavin also had no effect.

Preliminary results indicate that the alcohol precipitate factor is quite heat-labile. When the crude fraction obtained from liver extract was autoclaved for 6 hours at 120° and 15 pounds pressure, the activity was completely destroyed. Definite reduction in the potency of yeast has been observed due to autoclaving and heating dry at 120° for 3 days. Like the other factors in the vitamin B complex, the stability is somewhat dependent upon pH and a detailed report of the stability will be made in a later paper.

Concentration of Factor from Liver Extract—Attempts to use 80 and 90 per cent concentrations of alcohol to precipitate the factor from an aqueous solution of liver extract did not give consistent results. In the case of 90 per cent alcohol about one-half of the potency remained in the filtrate and one-half in the precipitate. We returned, therefore, to the use of a mixture of alcohol and ether for the first fractionation and acetone was used in the additional steps.

100 gm. of liver extract were dissolved in 200 cc. of H₂O and 1 liter of ethyl alcohol and 1200 cc. of ethyl ether were added with constant stirring. The precipitate was allowed to settle out and the supernatant liquid siphoned off. The precipitate was dissolved in 100 cc. of water and reprecipitated. After being washed with an ether-alcohol-water mixture, the precipitate was dissolved in 400 cc. of H₂O and allowed to stand for 24 hours, whereupon a light colored precipitate settled out, which was centrifuged off and washed with 25 cc. of H₂O. This precipitate was found to be inactive (Curve 2, Chart III), while the filtrate was active (Curve 1). The combined filtrate and washings were concentrated *in vacuo* at 50° to 80 cc. Upon the addition of 10 volumes of acetone, a dark gummy precipitate formed, which was active. The filtrate was inactive (Curve 3, Chart III). The precipitate was dissolved in 50 cc. of water and reprecipitated with 10 volumes

of acetone. After being washed with acetone, the precipitate was dissolved in 250 cc. of H_2O and allowed to stand, whereupon more insoluble material settled out, which was centrifuged off and washed with 25 cc. of H_2O . The precipitate was inactive (Curve 4, Chart III), whereas the filtrate was active (Curve 5). The filtrate was diluted to 300 cc. and 95 per cent alcohol added until the alcohol concentration reached 50 per cent, whereupon a light colored voluminous precipitate was obtained, which was washed in 50 per cent alcohol and reprecipitated. The combined filtrates were active (Curve 6), while the precipitate was inactive. The

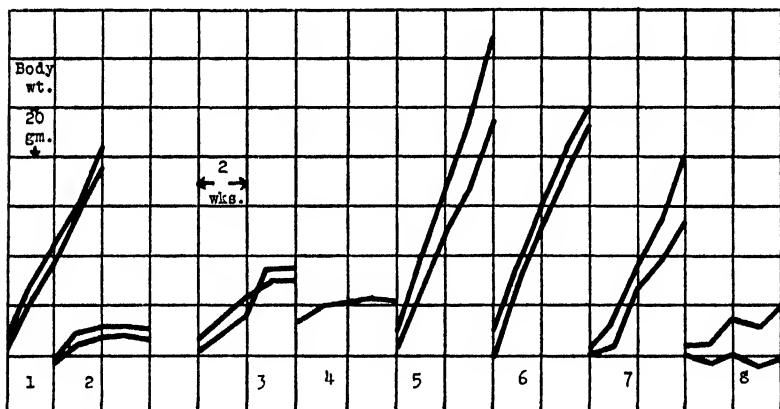


CHART III. Growth curves for rats on basal Ration K_{12} plus fractions prepared during the concentration of the alcohol-ether precipitate factor from liver extract.

alcohol was removed from the filtrate by vacuum distillation and the solution diluted to 1 liter. The solution was heated to 70° and the factor adsorbed by shaking with 10 gm. portions of norit until the solution was practically colorless. Five 10 gm. portions of charcoal were needed. The filtrate was inactive (Curve 8). When the charcoal was fed equivalent to 2 per cent of liver extract, good growth was obtained (Curve 7). Considerable difficulty has been encountered in attempts to remove the active substance from the charcoal. Other methods of concentration are now being studied.

DISCUSSION

A question of prime importance in describing a new dietary factor is its relation or possible identity with known essential factors. We have shown that the alcohol-ether precipitate factor cannot be flavin or vitamin B₂. Identity with vitamin B₄ is ruled out by the fact that our basal ration contains sufficient white corn to meet the vitamin B₄ requirements of the rat. Also, peanuts and brain tissue, known sources of vitamin B₄, are inactive.

Hogan and Richardson (22), using a sucrose ration which contained an irradiated alcohol extract of yeast as a source of vitamin B₁, produced two distinct deficiency symptoms—a denuding which was cured by flavins, and a general dermatitis which was cured by an alcoholic extract of corn-starch or by an ether extract of wheat germ. The relation of this factor to vitamin B₆ has been discussed by Birch and György (21). That the precipitate factor is distinct from vitamin B₆ is evidenced by the fact that an ether extract of wheat embryo, found by Hogan and Richardson to be active, failed to give any response on our ration, and by the fact that Birch, György, and Harris (5) have shown that corn is rich and liver extract is low in vitamin B₆.

Since the precipitate factor has such a profound effect on the growth of rats and since the body is so readily depleted of this factor, the idea presented itself that it might be an amino acid. Arginine and cystine, the two amino acids most easily destroyed by our method of purifying casein, proved to be inactive. Blood fibrin, which is rich in α -amino- β -hydroxybutyric acid, also failed to give a response. Finally, crude commercial casein, which is considered to be a complete protein for rats, was found to be inactive. Thus the evidence points quite clearly to the fact that this factor is not an amino acid. Furthermore, this factor is destroyed by autoclaving for 6 hours at pH 7, a property which is not characteristic of amino acids.

Choline, shown by McHenry (23) to be an accessory food factor, is also ruled out because of its inactivity.

Although evidence has been advanced that the factor described in this paper is distinct from vitamins B₁, B₂, B₄, B₆, and flavin, we must still consider its relation to certain additional factors which have been reported in the literature. Since we have found

the alcohol precipitate factor to be quite heat-labile, only those factors which appear to be thermolabile will be discussed.

In 1927 Williams and Waterman (24, 25) described a thermolabile factor in yeast necessary for pigeons. However, they demonstrated that rats did not need this factor. Of course this may merely mean that rats fed on the ration used by them did not require this material. Williams and Eddy (26) named this factor vitamin B₃ and later Eddy and coworkers (27) showed it to be necessary for chicks. Carter and O'Brien (28) have recently described the concentration of vitamin B₃ from liver and obtained two fractions—one of which was adsorbed on fullers' earth (flavin) and the other which remained in the filtrate. Although the factor described in this paper may have appeared in one of the above fractions, it is also possible that Carter and O'Brien were dealing only with flavin and vitamin B₂.

The relation of the alcohol precipitate factor to the casein factor described by Coward and coworkers (29) is not clear. It appears that they are not identical, since the latter workers were unable to extract their factor from yeast and the alcohol precipitate factor is found in yeast and is readily water-soluble. We also found crude casein to be a very poor source of our factor. It also appears that it is different from "physin" since Mapson (30) found physin to be readily soluble in acetone. The third factor of Hunt (31) may bear some relation to the factor described here. The relation of the additional factor described by Stiebeling (32) is also difficult to explain, since her work was done before the appreciation of the requirement of both flavin and vitamin B₂ in the B complex. Since the additional factor in the vitamin B complex described by Block and Hubbell (33) is adsorbed on Lloyd's reagent, it may be related to the alcohol precipitate factor, as we found it to be adsorbed on charcoal. However, their factor was prepared from rice polishings and we have not tested this material for the alcohol precipitate factor.

Jansen *et al.* (34), using a ration devoid of all vitamins of the B complex, obtained normal growth in rats when the ration was supplemented with an aqueous extract of yeast, from which they conclude that this extract must contain all unknown water-soluble vitamins which are necessary for normal growth in rats. The

alcohol-ether precipitate factor is undoubtedly one of the factors contained in this yeast extract.

The work on the precipitate factor presents many important aspects, the most interesting of which is its relationship to studies on vitamins B₁ and B₄. Since it is destroyed by autoclaving, autoclaved rations used for vitamin B₁ assay must be low in this factor, thereby limiting the growth and causing irregular appearance of symptoms. A number of workers have suggested that a small amount of vitamin B₁ must be added to a vitamin B₁-low ration to produce polyneuritis. Since the vitamin B₁ is generally added as yeast, it is probable that it is the alcohol-ether precipitate which is the limiting factor. Kline, Bird, Elvehjem, and Hart (19) experienced difficulty in producing a vitamin B₄ deficiency with a synthetic ration in which the known factors were supplied in concentrated form. They found, however, that this difficulty could be overcome by using yeast or liver extract as a source of these factors. Here again these crude supplements were furnishing the necessary alcohol-ether precipitate.

It is obvious that this factor must be taken into consideration in any attempt to produce pellagra in the rat. In the absence of this factor, complete failure of growth results, which would render impossible any attempt to deplete the animal of vitamin B₂. Robinson and Newton (35) were able to produce flavin and vitamin B₂ (P-P factor) deficiencies in rats only in the presence of carefully controlled amounts of yeast in the ration. Again the limiting factor was undoubtedly the alcohol-ether precipitate. We found, however, that it is extremely difficult to add the alcohol-ether precipitate factor without adding vitamin B₂ as a contaminant. Perhaps it was the difficulties mentioned above which prompted Birch, György, and Harris (5) to conclude that, "Rats do not need the human P.-P. (or canine blacktongue) factor in any significant amount, or are able to synthesise it."

Thus it is evident that much of the difficulty in the use of synthetic rations reported in the literature is due to a lack of this factor. Although we have demonstrated that the alcohol-ether precipitate factor is not identical with any of the known essential dietary factors, we have not given a definite name to this factor other than calling it the alcohol-ether precipitate factor. Our

suggestions for the designation of this factor are being submitted to the Committee on Nomenclature of the American Society of Biological Chemists.

SUMMARY

1. An essential dietary factor is described, which is distinct from the known dietary essentials.
2. This factor occurs in liver, yeast, and milk.
3. It is water-soluble, insoluble in the fat solvents, labile to autoclaving, and adsorbed on charcoal.
4. A method of concentrating the factor is described.

We are indebted to Dr. C. Nielsen of the Abbott Laboratories and Dr. David Klein of The Wilson Laboratories who supplied us with liver extract. We are also indebted to Dr. A. G. Hogan for sending us an ether extract of wheat germ which he had shown to be active in the prevention of dermatitis in rats.

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THE SPATIAL CONFIGURATION OF α -AMINO- β -HYDROXY-*n*-BUTYRIC ACID*

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Several months ago McCoy, Meyer, and Rose (1935) described the isolation and identification of α -amino- β -hydroxy-*n*-butyric acid as a cleavage product of proteins, and demonstrated that the new amino acid is an indispensable component of the diet. Since that time attention has been directed to the determination of the spatial configuration of the compound. It is the purpose of the present paper to outline these investigations and to suggest a simple name for the amino acid.

EXPERIMENTAL

Obviously, α -amino- β -hydroxy-*n*-butyric acid may exist in four optically isomeric forms. In our previous report (McCoy, Meyer, and Rose, 1935) we pointed out that the natural acid has a specific rotation of approximately -28° . Reduction with hydriodic acid and red phosphorus (*cf.* Abderhalden and Heyns (1934)) yielded an α -amino-*n*-butyric acid having "a slight but unmistakable *dextrorotation*." Unfortunately, it was not possible to determine accurately the specific rotation of the reduced product because of the small amount of material available at that time.

We have now repeated the reduction using slightly modified conditions. For this purpose, a mixture of 1 gm. of the hydroxy-amino acid, 0.3 gm. of red phosphorus, and 12 cc. of hydriodic acid (sp. gr. 1.96) was heated in a sealed tube at 150 – 155° for 7 hours. After cooling, the contents of the tube were diluted with 300 cc. of water and evaporated *in vacuo* to a volume of 3 cc. The

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latter was diluted to 250 cc. and treated with moist silver oxide for the removal of the iodide ion. The excess silver was precipitated with hydrogen sulfide, and the silver sulfide was removed by filtration. The solution was decolorized with a small amount of norit, and after filtration was concentrated *in vacuo* to about 2 cc. Absolute alcohol was added until a faint turbidity developed, and the solution was placed in a refrigerator for crystallization. The product was recrystallized twice by dissolving in the minimum amount of water and adding absolute alcohol to incipient precipitation. The following values were obtained on analysis.

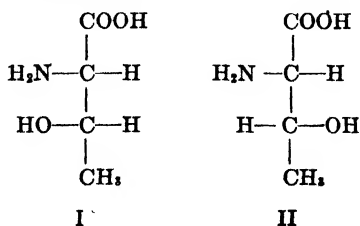
$C_4H_9NO_2$. Calculated. C 46.57, H 8.80, N 13.59
Found. " 46.77, " 8.69, " 13.41

A determination of the specific rotation in aqueous solution yielded the following result.

$$[\alpha]_D^{24} = \frac{+0.25^\circ}{2 \times (0.1495/5)} = +4.18^\circ$$

According to Oikawa (1925), natural α -amino-*n*-butyric acid has a specific rotation of $+8.05^\circ$. Fischer and Mouneyrat (1900) report values of $+8.0^\circ$ and -7.92° , respectively, for the two enantiomorphs prepared by the resolution of synthetic α -aminobutyric acid. Evidently, almost half of our compound had undergone racemization during the reduction; but the data demonstrate conclusively that the product was the *dextrorotatory* form of α -amino-*n*-butyric acid.

The above observations suffice to establish the spatial relationship of the groups around the α -carbon atom of our hydroxyaminobutyric acid. *d*- α -Amino-*n*-butyric acid, like all other naturally occurring α -amino acids thus far investigated, belongs to the *L* series (*cf.* Clough (1918) and Levene (1926)). Thus the hydroxyaminobutyric acid present in proteins must have one of the two possible configurations indicated in Formulas I and II.



Formula I is analogous to the structure of the sugar *l* (+)-erythrose, while Formula II corresponds to the configuration of *d* (-)-threose.¹

In order to establish which of the above represents the correct formula, advantage was taken of the observation of Dakin (1917) that oxidation of an α -amino acid (1 mole) with chloramine-T (1 mole) yields the corresponding aldehyde with 1 less carbon atom. According to this procedure, hydroxyaminobutyric acid should yield a lactic aldehyde whose spatial configuration could be identified by further oxidation to the lactic acid. For this purpose, 2 gm. of hydroxyaminobutyric acid were dissolved in 10 cc. of water and treated with a solution of 5 gm. of chloramine-T in 40 cc. of water. The temperature of the amino acid solution was about 30°, while that of the reagent was approximately 80°. The mixture was stirred vigorously for 15 minutes, and was then cooled in an ice bath until most of the toluene sulfonamide had crystallized. After filtration, the precipitate was washed with a small amount of ice water, and the washings were added to the main solution. The whole was diluted to 100 cc.

A portion of the above solution was tested for its optical rotation. It rotated strongly to the left, indicating that the oxidation had not proceeded to pyruvic aldehyde or acid. The biuret reaction was negative, showing the absence of hydroxyaminobutyric acid. As further proof that lactic aldehyde was actually produced, a sample of synthetic α -amino- β -hydroxy-*n*-butyric acid² was treated with chloramine-T, as outlined above, and the resulting product was transformed into the *p*-nitrophenylosazone according to the method of Dakin and Dudley (1913). After purification, the derivative was found to contain 24.58 per cent of nitrogen as compared with the theoretical value of 24.56 per cent. Obviously, this reaction is incapable of distinguishing between lactic aldehyde and pyruvic aldehyde, but that the latter was not obtained was demonstrated by the strong *levorotation* of the

¹ Divergent opinions concerning the direction and degree of rotation of *d*-threose are expressed in the literature, but Hockett (1935) reports that the sugar manifests an equilibrium rotation in water of approximately -12.4°.

² This racemic modification consisted of the natural product and its enantiomorph. It was synthesized in this laboratory by Dr. H. E. Carter and Mr. H. D. West, who will publish the procedure in the near future.

compound produced by the degradation of the natural hydroxyaminobutyric acid.

No attempt was made to isolate the lactic aldehyde formed from the natural hydroxyamino acid. Rather it was oxidized to the corresponding lactic acid, which was purified as its zinc salt. For this purpose, the solution was acidified with a few drops of hydrochloric acid and treated with bromine water until an excess had been added as indicated by the presence of an orange color after the flask has been allowed to stand in the sun. After 24 hours, the solution was concentrated *in vacuo* to 50 cc. and repeatedly extracted with ether. 500 cc. of the solvent were used. The ether was then removed *in vacuo*, and the residue was dissolved in 100 cc. of water. Basic zinc carbonate was added in excess, and the mixture was shaken for a few minutes and filtered. The filtrate was concentrated *in vacuo* to incipient crystallization and treated with an equal volume of alcohol. This not only promoted the crystallization of the zinc lactate, but prevented the separation of contaminating toluene sulfonamide. One recrystallization of the zinc lactate from the minimum quantity of water yielded 1.85 gm. of pure $\text{Zn}(\text{C}_3\text{H}_5\text{O}_3)_2 \cdot 2\text{H}_2\text{O}$, as compared with a theoretical yield of 2.35 gm., from 2 gm. of hydroxyaminobutyric acid. Analysis of the *anhydrous* salt gave the following values.

$\text{Zn}(\text{C}_3\text{H}_5\text{O}_3)_2$.	Calculated.	C 29.57, H 4.14, Zn 26.85
	Found.	" 29.90, " 4.26, " 26.74, 26.87

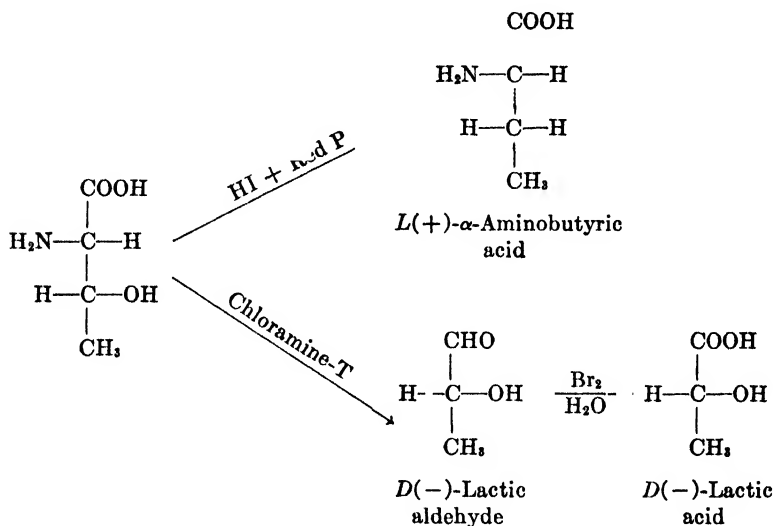
By the same procedure a second sample of zinc lactate was prepared from hydroxyaminobutyric acid, and the specific rotation of each (anhydrous salt) was determined. The results were as follows:

$$\begin{aligned} \text{Sample 1. } [\alpha]_D^{25} &= \frac{+0.22^\circ}{2 \times (0.1007/5)} = +5.46^\circ \\ &\quad \frac{+0.42^\circ}{2 \times (0.2619/10)} \quad +8.02^\circ \end{aligned}$$

Values reported in the literature for 2.5 per cent aqueous solutions range from $+8.0^\circ$ to $+8.2^\circ$ (*cf.* Cori and Cori (1929)).

It is well known that the direction of rotation of zinc lactate is opposite to that of the free lactic acid from which it is prepared

(cf. Freudenberg (1914); Cori and Cori (1929)). Thus the lactic acid obtained by the action of chloramine-T on natural hydroxy-aminobutyric acid is *levorotatory*, and hence belongs to the *D* series (cf. Levene (1926)). These facts demonstrate that the β -carbon possesses the *D* configuration, and that *our hydroxyamino-butyric acid is an L-D type corresponding in spatial structure to d(-)-threose*. The accompanying diagram illustrates the reactions involved.



These reactions definitely establish the spatial configuration of the new amino acid. Confirmatory evidence for the threo structure was obtained by an entirely different method of attack; namely, the transformation of the hydroxyamino acid into the corresponding dihydroxybutyric acid, and the identification of the latter as its phenylhydrazide. For this purpose, 2 gm. of natural hydroxyaminobutyric acid were dissolved in 20 cc. of water and treated with 4.9 gm. of barium nitrite monohydrate dissolved in 30 cc. of water. The solution was cooled in an ice bath. A slight excess of N sulfuric acid was added dropwise over a period of 30 minutes. After quantitative removal of the barium and sulfate ions, the solution was evaporated to a syrup *in vacuo*. Absolute alcohol was added twice and removed, and the product

was finally dissolved in 3 cc. of the same solvent. It is well known that nitrous acid does not induce a Walden inversion, and that a hydroxy acid so formed has the configuration characteristic of the amino acid from which it is derived (*cf.* Levene (1926)).

Attempts were made to crystallize the dihydroxy acid; but these efforts were unsuccessful because of the extreme solubility and the relatively small amount of the compound which was available. Therefore, the phenylhydrazide was prepared by adding 2.5 gm. of phenylhydrazine to the alcoholic solution of the dihydroxy acid (3 cc.) and heating the mixture for 30 minutes at 75° (*cf.* Glattfeld and Woodruff (1927)). Upon standing over-

TABLE I
Phenylhydrazides of Dihydroxybutyric Acids

Sample No.	Phenylhydrazide of	Melting point	Nitrogen*
		°C.	per cent
1	Dihydroxy acid from natural amino acid	103.5-104	12.40
2	" " " synthetic " "	129.5-131	12.25
3	<i>l</i> -Threodihydroxy acid (Glattfeld)	103.5-104	12.31
4	<i>d</i> -Threodihydroxy " "	102 -104	
5	<i>dl</i> -Threodihydroxy " (recrystallized mixture of Samples 3 and 4)	129.5-130†	12.42
6	<i>dl</i> -Erythrodihydroxy acid (Glattfeld)	103	
7	Equal quantities of Samples 1 and 3 (for mixed m. p.)	103 -104	

* Theoretical N for $C_{10}H_{14}N_2O_3 \cdot H_2O$ is 12.28 per cent.

† Glattfeld and Woodruff (1927) report a melting point of 129.5° for the phenylhydrazide of *dl*-threodihydroxybutyric acid.

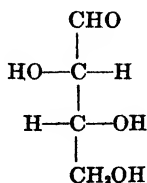
night in a refrigerator, crystals were obtained. These were recrystallized three times from the minimum amounts of absolute alcohol.

In a similar fashion, the phenylhydrazides of *d*- and *l*-threodihydroxybutyric acids, *dl*-erythrodihydroxybutyric acid,³ and the *dl*-dihydroxy acid derived from *dl*-hydroxyaminobutyric acid²

³ Professor J. W. E. Glattfeld of the University of Chicago kindly furnished the samples of *d*- and *l*-threo- and *dl*-erythrodihydroxybutyric acids. It is a pleasure to acknowledge our indebtedness to Professor Glattfeld for his cordial cooperation. The prefixes *d*- and *l*- as used with the threo acids denote the *direction* of rotation, and not the series to which the compounds belong.

were prepared. In addition, equal amounts of the phenylhydrazides of *d*- and *l*-threodihydroxybutyric acids were recrystallized together, thereby providing a racemic preparation. Hence, six phenylhydrazides were available for comparison. The melting points and analytical data are presented in Table I, and demonstrate that natural hydroxyaminobutyric acid yields a dihydroxy acid whose phenylhydrazone is identical with the like derivative of one of the active (*d*- or *l*-) threodihydroxybutyric acids. Furthermore, the synthetic amino acid, consisting of the natural product and its enantiomorph, yields a dihydroxy acid whose phenylhydrazone is identical with that of *dl*-threodihydroxybutyric acid. The phenylhydrazone of the latter is a racemic compound rather than a racemic mixture, inasmuch as its melting point is higher than that of its components. Oddly enough, the phenylhydrazides of the *d*- and *l*-threo acids and of the *dl*-erythro acid melt within the same range.

The fact that nitrous acid transforms natural hydroxyaminobutyric acid into a threodihydroxybutyric acid establishes the spatial configuration of the amino acid; for, as stated above, it has been shown repeatedly in this laboratory that reduction of the hydroxyamino acid yields *L*(+)- α -aminobutyric acid. *The only configuration which can account for these findings is that shown in Formula II.* Thus the observations upon the dihydroxy acids are in complete accord with the data obtained by the degradation of the amino acid with chloramine-T. *It is proposed that henceforth natural α -amino- β -hydroxy-*n*-butyric acid be known as *d*(-)-threonine,⁴ inasmuch as it possesses a spatial configuration analogous to that of *d*(-)-threose (Formula III).*



d(-)-Threose

III

⁴ The *d* here refers, of course, to the *series* just as it does in the classification of sugars. With equal propriety the amino acid could be known as *D*(-)-threonine.

The above deduction concerning the spatial structure of threonine, as determined by its relation to threodihydroxybutyric acid, obviously does not necessitate previous knowledge as to the configuration of the dihydroxy acid. Having first established the relative positions of the groups attached to the α -carbon atom of the amino acid, the configuration of the β position of the latter follows inevitably from the fact that a threodihydroxybutyric acid was obtained. On the other hand, our observations provide important information regarding the spatial structure of the isomeric threodihydroxy acids. Braun (1930) demonstrated the relation of *dl*-threo- and *dl*-erythro-dihydroxybutyric acids to the tartaric acids; and Glattfeld and Chittum (1933) accomplished the resolution of the threo acids. But the series to which each of the latter belongs appears not to have been established.

In order to obtain information of this sort, comparisons of the specific rotations of the threo acid phenylhydrazides were undertaken. The results demonstrate that the phenylhydrazide of the dihydroxy acid derived from threonine and the phenylhydrazine of Glattfeld's *l*-threodihydroxybutyric acid are identical. The specific rotations (in aqueous solution) are shown below.

Phenylhydrazide of Dihydroxybutyric Acid Derived from d(-)-Threonine—

$$[\alpha]_D^{25} = \frac{-0.25^\circ}{2 \times (0.0389/5)} = -16.07^\circ$$

Phenylhydrazide of l-Threodihydroxybutyric Acid (Glattfeld)—

$$[\alpha]_D^{25} = \frac{-0.66^\circ}{2 \times (0.1015/5)} = -16.25^\circ$$

Thus the dihydroxy acid which is *levorotatory*, and yields a *levorotatory* phenylhydrazide, is *d*(-)-threodihydroxybutyric acid. Its enantiomorph is *l*(+)-threodihydroxybutyric acid.

SUMMARY

A study has been made of the spatial configuration of natural α -amino- β -hydroxy-*n*-butyric acid. The results demonstrate that this constituent of proteins is an *L-D* compound analogous in struc-

ture to *d*(-)-threose. Because of this relationship, *the amino acid has been named d*(-)-*threonine*.

It has been shown that the *dextro*- and *levothreodihydroxy*-butyric acids described in the literature are *l*(+) and *d*(-) compounds, respectively.

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AN IMPROVED METHOD FOR THE PREPARATION OF XYLULOSE AND RIBULOSE

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In 1927 it was discovered¹ that glyceric aldehyde is transformed into dihydroxyacetone, to the extent of about 50 per cent, on treatment with boiling pyridine. On extending the reaction to glucose, the formation of fructose was observed,² and it was claimed that under these conditions the formation of the epimeric aldose does not take place. Similarly, by the action of boiling pyridine on *d*-xylose, there is obtained a ketopentose which has been variously designated as *d*-xyloketose,³ threo-2-ketopentose,⁴ and, erroneously, as arabinulose.⁴ For simplicity it would seem desirable to term the ketose "*d*-xylulose," in conformity with the nomenclature accepted for other keto sugars.

In the same way, *l*-xylulose has been prepared⁵ from *l*-xylose (and found to be identical with the ketose isolated by Levene and La Forge⁶ from urine in cases of pentosuria), and the formation of *d*-^{7,8} and *l*-ribulose⁷ from *d*- and *l*-arabinose respectively has been demonstrated. *d*-Ribulose was identified as its *o*-nitrophenylhydrazone,⁸ but the properties of the pure free keto sugars obtained by this method have not as yet been described, though pure *l*-ribulose⁴ has been prepared from ribitol.

¹ Fischer, H. O. L., Taube, C., and Baer, D., *Ber. chem. Ges.*, **60**, 479 (1927).

² Danilov, S., Venus-Danilova, E., and Schantorovitsch, P., *Ber. chem. Ges.*, **63**, 2269 (1930).

³ Schmidt, O. T., and Treiber, R., *Ber. chem. Ges.*, **66**, 1765 (1933).

⁴ Reichstein, T., *Helv. chim. acta*, **17**, 996 (1934).

⁵ von Vargha, L., *Ber. chem. Ges.*, **68**, 18 (1935).

⁶ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **18**, 319 (1914).

⁷ Schmidt, O. T., and Heintz, K., *Ann. Chem.*, **515**, 77 (1934).

⁸ Glatthaar, C., and Reichstein, T., *Helv. chim. acta*, **18**, 80 (1935).

In the record of an attempt by Levene and Hill⁹ to study its mechanism, these authors state, "The reaction was followed by titration of the total sugar present by the Lehmann modification of the Fehling's titration and the aldoses by Willstätter and Schudel's hypiodite titration as modified by Goebel. We were unable to detect any conversion of xylose to ketoxylose by this method."¹⁰ It is now known that this was due to the ready reaction⁷ of xylulose with the hypiodite reagent. Presumably owing to some misunderstanding, Schmidt and Heintz⁷ misinterpreted the above statement as indicating that Levene and Hill considered xylulose had not been formed.

Contrary to the general statement of Danilov *et al.*,² Schmidt and Heintz⁷ have observed the formation of *d*-lyxose concomitantly with the ketose from *d*-xylose. It was conjectured that the crude ribulose isolated from treatment of arabinose might well contain some ribose, though they were unable to demonstrate its presence.

The present methods of isolating the ketopentoses are slow and, if desired in large quantities, are also costly, since they involve the use of substituted phenylhydrazines, resulting in relatively poor yields of product. With the object of rendering xylulose readily available for both biological and chemical study, we have developed a quick and cheap method for the isolation of this sugar. The method consists in separating the sugars contained in the crude reaction mixture by condensing with acetone.

Provided that the reaction is permitted to proceed to completion, the crude product consists of three substances, the acetone derivatives of xylose, lyxose, and xylulose. It was known that xylose forms a diacetone derivative,¹¹⁻¹³ xylulose a monoacetone compound,¹⁴ and lyxose has now been found likewise to form a monoacetone derivative. The monoacetone derivatives may be separated from the diacetone xylose by fractional distillation.

⁹ Levene, P. A., and Hill, D. W., *J. Biol. Chem.*, **102**, 563 (1933).

¹⁰ The italics are ours.

¹¹ Freudenberg, K., and Svanberg, O., *Ber. chem. Ges.*, **55**, 3239 (1922).

¹² Svanberg, O., and Sjöberg, K., *Ber. chem. Ges.*, **56**, 863 (1923).

¹³ Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

¹⁴ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 603 (1934).

The monoacetone xylulose is separable from that of lyxose by virtue of its crystallizing properties.

Ribose yields monoacetone ribose together with a little of its anhydride,¹⁵ whereas arabinose¹⁶ and ribulose⁴ give diacetone derivatives and the monoacetone derivative may be separated from the diacetone compounds by fractional distillation.

Since the properties of certain of these compounds had been inadequately described, it was found necessary to repeat their

TABLE I
Some Properties of Isopropylidene Pentoses

Substance	Approximate b.p. at 0.1 mm.	n_D^{25} (superfused substance)	M.p.	$[\alpha]_D^{25}$ (in acetone)
	$^{\circ}\text{C.}$		$^{\circ}\text{C.}$	degrees
Diacetone <i>d</i> -xylose.....	78	1.4587	44-45	+19.5
Monoacetone <i>d</i> -lyxose... (110)	(110)	1.4724	79-80	+26.0
Monoacetone <i>d</i> -xylulose.	110	1.4672	70-71*	+1.7
Diacetone <i>l</i> -arabinose...	70	1.4520	42	± 0.1
Anhydromonoacetone <i>d</i> -ribose.....	50-57	1.4565	61	-64.7
Monoacetone <i>d</i> -ribose...	109	1.4684	(Only obtained as syrup, mixture of α and β forms?)	-27.4 (For typical material, varies according to proportions of α and β form present)

* Previously reported as 50-52° (Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 603 (1934)).

preparation in order to study such properties as would be of value in the present investigation. The results, most of which appear to be new, are given in Table I. From Table I it is seen that when an acetone derivative of a pentose is obtained in crystalline form, observation of the refractive index and the specific rotation (to-

¹⁵ Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933).

¹⁶ Fischer, E., *Ber. chem. Ges.*, **28**, 1163 (1895).

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gether with the elementary composition) suffices to give a reliable indication of the nature of the constituent pentose. If the material is not obtained crystalline, however, such observations do not yield precise information, especially since the possibility is not excluded that the monoacetone derivatives of lyxose and ribose may exist in both α and β modifications, thus rendering the value of the specific rotation (in acetone) deceptive.

TABLE II

Specific Rotations (in Water) of Pentoses and Their Isopropylidene Derivatives

Pentose	Diacetone derivative	Monoacetone derivative	Free sugar (equilibrium)
	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
<i>d</i> -Xylose.....	+13*	-19†	+19‡
<i>d</i> -Xylulose.....		-6.5	-33§
<i>d</i> -Lyxose.....		+18.7 (Final)	-14
<i>l</i> -Arabinose.....	+5¶	+128.8 (For compound + $\frac{1}{2}$ H ₂ O)**	+105‡
<i>l</i> -Ribulose.....	(+107 †† (In acetone))		+16††
Anhydro- <i>d</i> -ribose.....		-59.4	
<i>d</i> -Ribose.....		-12.5 (Final)	-19††

* Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

† Svanberg, O., and Sjöberg, K., *Ber. chem. Ges.*, **56**, 863 (1923).

‡ Hudson, C. S., and Yanowsky, E., *J. Am. Chem. Soc.*, **39**, 1013 (1917).

§ Schmidt, O. T., and Treiber, R., *Ber. chem. Ges.*, **66**, 1765 (1933).

|| Ruff, O., and Ollendorff, G., *Ber. chem. Ges.*, **33**, 1798 (1900).

¶ Fischer, E., *Ber. chem. Ges.*, **28**, 1163 (1895).

** Ohle, H., and Behrend, G., *Ber. chem. Ges.*, **60**, 810 (1927).

†† Reichstein, T., *Helv. chim. acta*, **17**, 996 (1934).

‡‡ Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **42**, 2469 (1909).

When, however, the course of hydrolysis of such material is followed by observation of the change in specific rotation, further insight is gained into its composition. For convenience, the specific rotations (in water) of the pentoses and their acetone derivatives are presented in Table II.

It may be mentioned that the yield of pure recrystallized mono-

acetone xylulose actually isolated amounted to some 18 per cent¹⁷ (by weight) of the crude mixture of acetone compounds. The method does not seem appropriate for the preparation of lyxose without further modification, although the presence of some 14 per cent of monoacetone lyxose in the crude acetone compound mixture was revealed by the hydrolysis data and crystalline lyxose *p*-bromophenylhydrazone was actually isolated.

Similarly, the method may be applied to the preparation of ribulose from arabinose but does not seem advantageous for the preparation of ribose. The yield of diacetone ribulose amounted to some 28 per cent¹⁷ (by weight) of the crude mixture of acetone compounds.

EXPERIMENTAL

Recrystallization of Pentoses

d-Xylose—10 gm. were dissolved in 100 cc. of boiling 95 per cent ethyl alcohol and the solution cooled. M.p. 143°; $[\alpha]_D^{23} = +19.2^\circ$ (equilibrated in H₂O).

d-Lyxose—10 gm. were dissolved in 50 cc. of boiling absolute ethyl alcohol and the solution was cooled. M.p. 101°; $[\alpha]_D^{23} = -14.3^\circ$ (equilibrated in H₂O).

l-Arabinose—10 gm. were dissolved in 200 cc. of boiling 95 per cent ethyl alcohol and the solution was cooled. M.p. 156°; $[\alpha]_D^{20} = +104^\circ$ (equilibrated in H₂O).

d-Ribose—This was recrystallized from the minimum of absolute ethyl alcohol. M.p. 85°; $[\alpha]_D^{25} = -19.3^\circ$ (equilibrated in H₂O).

Condensation of Pentoses with Acetone

The method of condensing the various pentoses with acetone was in every case identical, in order to obtain comparable results.

10 gm. of dry, finely powdered, recrystallized sugar were shaken at room temperature (25°) with a mixture of 20 gm. of anhydrous copper sulfate, 200 cc. of acetone (analytical reagent, except for lyxose and ribose, in which cases "acetone from bisulfite" was

¹⁷ These yields are minimum rather than the maximum possibly obtainable, since more material could be isolated by refractionation of impure fractions.

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employed), and 0.2 cc. of concentrated sulfuric acid until all the sugar had dissolved (approximately 48 hours). The mixture was now filtered and the product isolated as described for monoacetone uridine,¹⁸ giving a colorless or very pale yellow syrup which was dissolved in dry ether and freed from a trace of insoluble material by filtration.

The rapid method for condensation of sugars with acetone, which was recently introduced by Bell,¹⁹ is not recommended for use with the rarer pentoses, owing to the poor yield (70 per cent).

Purification of the crude acetone compound by dissolution in dry ether and filtration should never be omitted; xylose, for instance, is sufficiently soluble in diacetone xylose to be present in the crude product when the condensation with acetone has not been permitted to proceed to completion. The xylose is mainly precipitated by the addition of dry ether and the rest is left as a still residue on distillation under a high vacuum.

Pure Monoacetone d-Xylulose—This was prepared as previously described¹⁴ and recrystallized as described later. By recrystallization the melting point was raised from 50–52° to 70–71° and the purified substance was not hygroscopic. In the superfused state it had $n_D^{25} = 1.4672$ and the crystals displayed the following specific rotations.

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{2 \times 4.040} = +1.7^\circ \text{ (in acetone)}$$

$$[\alpha]_D^{25} = \frac{-0.26^\circ \times 100}{2 \times 2.004} = -6.5^\circ \text{ (in water, unchanged in presence of trace of oxalic acid or ammonia)}$$

4.190 mg. substance: 7.730 mg. CO₂ and 2.780 mg. H₂O

C₈H₁₄O₆. Calculated. C 50.50, H 7.4

Found. " 50.30, " 7.4

Experiments with pure *d-lyxose* revealed that, on condensation with acetone, this sugar gives a monoacetone derivative—as was predicted by Levene and Stiller¹⁵ on theoretical grounds. It is of interest that this substance shows a pronounced tendency to undergo autocondensation on heating, thus resembling the presumably structurally related trimethyl lyxofuranose.²⁰ Use of

¹⁸ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 113 (1934).

¹⁹ Bell, D. J., *J. Chem. Soc.*, 1874 (1935).

²⁰ Bott, H. G., Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 658 (1930).

this property was made in the partial separation of lyxose from xylose and xylulose.

*Pure Monoacetone d-Lyxose*²¹—This was prepared as above and was isolated as a colorless syrup which rapidly crystallized to an almost solid mass. It was recrystallized by dissolving in the minimum of dry ether, adding pentane to faint opalescence, nucleating, and allowing to stand in the refrigerator overnight. The recrystallized substance, which was not hygroscopic, had a melting point of 79–80°, $n_D^{25} = 1.4724$ (in the superfused state), and the following specific rotations.

$$[\alpha]_D^{25} = \frac{+1.10^\circ \times 100}{2 \times 2.118} = +26.0^\circ (\text{in acetone})$$

$$[\alpha]_D^{25} = \frac{+0.38^\circ \times 100}{2 \times 1.018} = +18.7^\circ (\text{in water, after adding 1 drop concentrated ammonia})$$

5.192 mg. substance: 9.600 mg. CO₂ and 3.430 mg. H₂O

C₅H₁₀O₅. Calculated. C 50.50, H 7.4

Found. " 50.42, " 7.4

The substance may be distilled in a high vacuum only if the distillation be rapid. With slow distillation it undergoes change to a substance of higher boiling point than that of a monoacetone pentose and, on cooling, solidifies to a hard, pale yellow glass in the distilling flask. This glassy product had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+3.21^\circ \times 100}{2 \times 2.182} = +73.6^\circ (\text{in acetone})$$

Hydrolysis of Acetone Derivatives of Pentoses

Monoacetone Xylulose—Preliminary experiments disclosed that, as with fructose, the use of mineral acids leads to considerable decomposition of the ketose. Recourse was therefore had to oxalic acid²² as hydrolyst. The concentration of acid finally

²¹ Some preliminary experiments on the preparation of acetone lyxose were performed in this laboratory by Dr. I. E. Muskat, under the direction of the senior author of the present communication.

²² Düll, G., *Chem.-Ztg.*, **19**, 166, 216 (1895). Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, **4**, 221 (1931). Hibbert, H., and Percival, E. G. V., *J. Am. Chem. Soc.*, **52**, 3995 (1930).

selected was 0.2 N, as the hydrolysis proceeds too slowly with 0.1 N aqueous oxalic acid.

A solution (approximately 2 per cent) of the recrystallized acetone compound in 0.2 N oxalic acid was prepared ($[\alpha]_D^{20} = -6.2^\circ$), and 8 cc. portions of this solution were placed in tubes which were then sealed and heated in a water bath maintained at 65° . After the elapse of various intervals of time a tube was removed, rapidly cooled in ice to room temperature, and the specific rotation observed. The results are given in Fig. 1.

After 2 hours at 65° the substance was only hydrolyzed to the

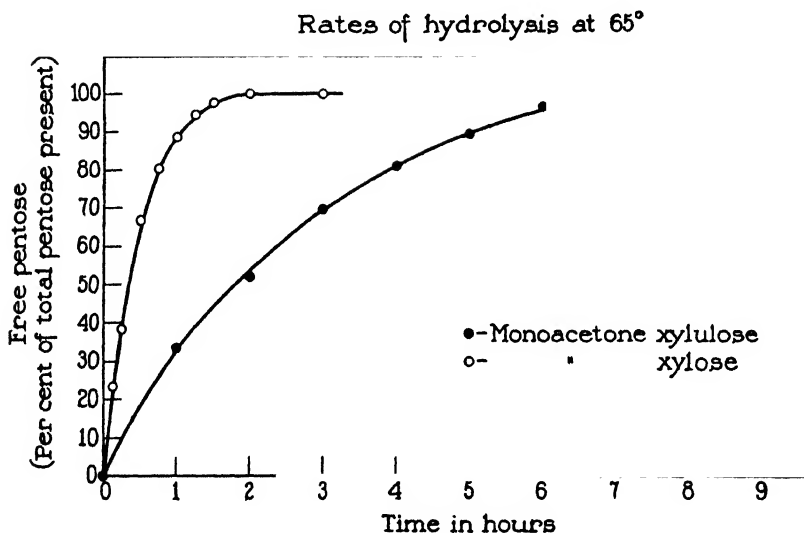


Fig. 1

extent of 52 per cent and 8 hours were required for complete hydrolysis, the solution then having $[\alpha]_D^{25} = -33.2^\circ$ (calculated as pentose). The isolation of the sugar is described later.

Diacetone Xylose—At room temperature (25°) the hydrolysis of a 2.422 per cent solution of dry, recrystallized diacetone xylose in 0.2 N aqueous oxalic acid had proceeded quantitatively to the stage of monoacetone xylose after 40 minutes. No further measurable hydrolysis had occurred after an additional 5 hours at room temperature, but after 24 hours a definite hydrolysis to xylose (to the extent of some 25 per cent) was observable.

When the reaction was performed at 65°, hydrolysis had proceeded quantitatively to xylose after 2 hours (see Fig. 1).

Monoacetone Ribose and Monoacetone Lyxose—Under identical conditions, the rates of hydrolysis of monoacetone ribose and monoacetone lyxose were practically the same as for monoacetone xylose.

Diacetone Arabinose—The hydrolysis of diacetone arabinose is of interest since at 65° it proceeds at approximately the same rate as for the *monoacetone* derivatives of the other aldopentoses. No appreciable formation of monoacetone arabinose could be detected by observation of the change in specific rotation during hydrolysis and the reaction appeared to proceed directly from the diacetone derivative to free arabinose.²³ This result may, however, be deceptive.

Application of Hydrolysis Results—It thus becomes a simple matter to detect the presence of even a trace of diacetone xylose in monoacetone xylulose or monoacetone lyxose. If the specific rotation (of a solution in 0.2 N oxalic acid) changes in the negative direction during 40 minutes at room temperature, diacetone xylose was present. Similarly, if there is a change in the negative direction between samples heated at 65° for 2 and 8 hours, monoacetone xylulose is present.

Analysis of Crude Xylulose Fraction (Through Acetone Compounds)

75 gm. of dry, finely powdered *d*-xylose were treated with 750 cc. of dry pyridine in the usual manner.³ The product was dissolved in 150 cc. of 95 per cent ethyl alcohol and the solution kept in the refrigerator overnight, giving 48.5 gm. of unchanged crystalline xylose. Further removal of xylose by treatment of the alcoholic solution with dry ether was omitted, since this is slow and is unnecessary when the following method of purification is employed.

25.5 gm. of colorless, crude xylulose syrup were condensed with acetone (analytical reagent) by the general method already described. The final ether solution obtained was evaporated to a colorless or very pale yellow syrup (yield, 27 gm.) which was

²³ Svanberg, O., and Bergmann, S. W., *Ark. Kemi, Mineral. o. Geol.*, **9**, No. 3, 1 (1923).

fractionated by distillation under a high vacuum. The fractions obtained are described in Table III.

Fraction 1 (Table III) had a composition corresponding approximately to that of a diacetone pentose, and will be referred to as the diacetone fraction. It crystallized to a considerable extent on nucleation with pure diacetone xylose.

Fractions 2 and 3 (Table III) had compositions corresponding approximately to that of a monoacetone pentose, and will be referred to as the monoacetone fraction.

Examination of Diacetone Fraction—The diacetone fraction contained, besides diacetone xylose, a small proportion of a substance having a lower refractive index and a negative specific rotation (in acetone), which has not yet been identified. After removal of the first crop of diacetone xylose, the syrupy mother liquor (78 gm., from several experiments) was distilled in a high vacuum, giving 1.6 gm. of a first fraction boiling at 41.5–49° at 0.1 mm. (bath temperature, 48–55°) and having $n_D^{25} = 1.4414$ and $[\alpha]_D^{25} = -47.8^\circ$ (in acetone). Its composition agreed with that of a diacetone pentose. A 2 per cent solution in 0.2 N oxalic acid had $[\alpha]_D^{25} = -45.5^\circ$, which remained unchanged during 40 minutes at room temperature, indicating the absence of any appreciable amount of diacetone xylose. On heating at 65° it had $[\alpha]_D^{25} = 0$ (2 hours), changing to -3.7° (8 hours). The solution was now strongly reducing to boiling Fehling's solution.

Examination of Monoacetone Fraction—Fractions 2 and 3 (Table III) both crystallized to a considerable extent when nucleated with a crystal of pure monoacetone xylulose¹⁴ prepared from xylulose regenerated from the *p*-bromophenylhydrazone.

But little variation was observable in the properties of the second and third fractions obtained in a number of different experiments. The properties of some of the monoacetone fractions obtained are recorded in Table IV. In each case the material was sufficiently pure to crystallize on nucleation with authentic monoacetone xylulose.

Monoacetone Xylulose—A total of 55 gm. of such material, obtained by combining the monoacetone fractions from several experiments, was purified once by distillation under a high vacuum. The main fraction (45 gm.) boiling at 110–118° at 0.1 mm. (bath temperature, 113–126°) was collected as a colorless syrup having

$n_D^{25} = 1.4666$ and $[\alpha]_D^{25} = +2.5^\circ$ (in acetone). It was nucleated with monoacetone xylulose and the crystalline mass separated from adhering syrup by trituration with the minimum volume of dry ether. The slightly sticky crystals (26 gm.+) were filtered off and recrystallized in the following way.

10 gm. of the slightly impure crystals were dissolved in the minimum (about 50 cc.) of dry ether at room temperature (or with

TABLE III
Fractionation of Acetone-Pentose Mixture

Fraction No.	Bath temperature	Temperature (Pressure 0.1 mm.)	Weight	n_D^{25}	$[\alpha]_D^{25}$ (in acetone)
	$^\circ\text{C.}$	$^\circ\text{C.}$	gm.		degrees
1	85-100	80- 87	12.0	1.4572	+6.3
2	112-120	105-111	5.0	1.4665	+2.7
3	120-130	111-118	5.6	1.4688	+3.4
Still residue.....	(Dark brown glass, soluble in acetone)		4.3		

TABLE IV
Properties of Some Monoacetone Fractions

n_D^{25}	$[\alpha]_D^{25}$ (in acetone)
	degrees
1.4661	+3.1
1.4689	+3.1
1.4675	+2.6
1.4662	+3.6
1.4689	+3.7
1.4654	+3.4
1.4678	+3.6

gentle warming); the solution was nucleated with a crystal of monoacetone xylulose and then allowed to stand in the refrigerator. The product rapidly crystallized (in crystals several cm. long). A further crop was obtained by addition of pentane (40 cc.) to faint opalescence and again standing in the refrigerator. The total yield of recrystallized material was 8 gm. The pure substance, which was not hygroscopic, had a melting point of 70-71°,

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n_D^{25} 1.4671 (superfused substance), and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.13^\circ \times 100}{2 \times 4.002} = +1.6^\circ \text{ (in acetone) .}$$

Its composition was as follows:

4.014 mg. substance: 7.405 mg. CO_2 and 2.680 mg. H_2O

$\text{C}_5\text{H}_{10}\text{O}_5$. Calculated. C 50.50, H 7.4

Found. " 50.30, " 7.5

On admixture with an authentic specimen of monoacetone xylulose (m.p. 70–71°) the melting point remained unaltered.

TABLE V

Fractionation of Monoacetone Material after Partial Removal of Monoacetone Xylulose

Fraction No.	Bath temperature	Temperature (Pressure 0.1 mm.)	Weight	n_D^{25}	$[\alpha]_D^{25}$ (in acetone)	From hydrolysis data	
						Monoacetone lyxose (approximate)	Diacetone xylose (approximate)
	°C.	°C.	gm.		degrees	per cent	per cent
1	98–101	88–94	5.3	1.4625	+4.5	67	33
2	102–108.5	95–102.5	7.9	1.4672	+1.9	56	9
3	109–117	103–107	10.6	1.4685	+4.3	76	0
4	120–140	110–126	3.2	1.4711	+7.5	100	0
Still residue. . . .	Dark brown gum		2.8				

Hydrolysis of Crystalline Monoacetone Xylulose—2 gm. of dry, recrystallized monoacetone xylulose and 0.9 gm. of anhydrous oxalic acid were dissolved in water and the solution diluted to 100 cc. with water. The solution was then heated at 65° during 8 hours.

Finely powdered calcium carbonate was now added, with shaking, until the solution was neutral to Congo red, the mixture filtered, and the calcium salts well washed with distilled water. The filtrate and washings were combined and evaporated to a colorless syrup which was extracted several times with absolute ethyl alcohol. A trace of insoluble material was filtered off and

the filtrate was evaporated to dryness under diminished pressure. Two portions of benzene were added and evaporated off, giving a colorless syrup (yield, 1.5 gm.) having the specific rotation given previously.

Monoacetone Lyxose—The syrupy mother liquor from the crystalline monoacetone xylulose was now examined. A total of 29.8 gm. of such material was fractionated by distillation under a high vacuum, the results being given in Table V. After complete hydrolysis with 0.2 N oxalic acid at 65°, a sample of Fraction 4 had $[\alpha]_D^{25} = -14.7^\circ$ (calculated as pentose) and therefore appeared to be almost pure monoacetone lyxose. This was verified as follows: 1 gm. of the material was dissolved in 0.2 N oxalic acid, the solution diluted to 50 cc. with 0.2 N oxalic acid, and the specific rotation observed immediately. The solution was now heated at 65° overnight. On cooling, it had $[\alpha]_D^{25} = -14^\circ$ (calculated as pentose). The sugar was isolated as described for xylulose from crystalline monoacetone xylulose.

The colorless syrup was now dissolved in absolute methyl alcohol and a solution of *p*-bromophenylhydrazine (the calculated weight) in absolute methyl alcohol was added. The *p*-bromophenylhydrazone commenced crystallizing immediately, but, to insure complete reaction, the solution was heated on the steam bath for a few minutes. On cooling, the mixture set to a solid mass of colorless crystals which, after one recrystallization from boiling water, had a melting point of 155–157° and the following composition.

4.505 mg. substance:	6.830 mg. CO ₂	and 1.910 mg. H ₂ O
7.300 " " "	: 0.571 cc. N ₂	(757 mm. at 26°)
5.575 " " "	: 3.290 mg. AgBr	
C ₁₁ H ₁₁ O ₄ N ₂ Br.	Calculated.	C 41.37, H 4.7, N 8.78, Br 25.05
	Found.	" 41.34, " 4.7, " 8.89, " 25.12

It had the following specific rotation

$$[\alpha]_D^{25} = \frac{+0.72^\circ \times 100}{2 \times 1.044} = +34.5^\circ \text{ (in dry pyridine, 5 minutes after admixture)}$$

changing to +14.8° (24 hours) and +10.0° (115, 139, and 165 hours) at room temperature. Had the material been the *p*-bromophenylhydrazone of either *d*-xylose or *d*-xylulose, the final specific rotation would have been negative.

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The values are in good agreement with those given by Levene and La Forge⁶ for *d*-lyxose *p*-bromophenylhydrazone in pyridine; namely, $[\alpha]_D^{25} = +31.8^\circ$ (10 minutes) changing to $+7.8^\circ$ (24 hours). (These figures are both approximations, slightly low in value, since Levene and La Forge expressed their concentration in gm. per 10 cc. of solvent instead of gm. per 10 cc. of solution.)

Action of Pyridine on l-Arabinose

75 gm. of dry, finely powdered *l*-arabinose (recrystallized from 95 per cent ethyl alcohol) were added to 750 cc. of dry pyridine. After standing overnight at room temperature with exclusion of

TABLE VI
Fractionation of Acetone-Pentose Mixture

Fraction No.	Bath temperature	Temperature (Pressure 0.1 mm.)	Weight	n_D^{25}	$[\alpha]_D^{25}$ (in acetone)	Composition found	
						Carbon	Hydrogen
	$^\circ\text{C.}$	$^\circ\text{C.}$	gm.		degrees	per cent	per cent
1	66	59- 61	5.9	1.4420	+84.5	58.17	8.3
2	66- 70	59- 61	2.7	1.4447	+71.5	57.24	8.2
3	67- 68	58	3.2	1.4472	+48.6	57.49	7.7
4	69- 83	53- 63	2.4	1.4512	+19.7	56.90	8.0
5	91-100	69- 88	0.5	1.4561	+12.8	57.66	8.5
6	118-128	108-111	5.2	1.4663	+12.8	51.55	7.6
Still residue.....	Light brown glass, soluble in acetone		3.4				

atmospheric moisture all the sugar had not dissolved. The mixture was now heated on the boiling water bath until most of the sugar had dissolved and was then boiled gently for 4 hours under a reflux condenser.

The pyridine was then removed as described for the preparation of crude xylulose,³ giving a thick syrup which was allowed to stand overnight at room temperature. Much of the unchanged arabinose had now crystallized out. The mixture was triturated with absolute ethyl alcohol, the colorless crystals filtered off, washed with a little absolute ethyl alcohol and then dry ether, and dried. Weight, 36 gm.

The mother liquor and washings were united and evaporated under diminished pressure to a thick gum weighing about 39 gm. This was again allowed to stand overnight at room temperature, whereupon a further crop of *l*-arabinose (19 gm.) was obtained.

The mother liquor and washings were united and evaporated to a thick gum weighing about 20 gm. and having $[\alpha]_D^{25} = +38^\circ$ (in water). This was condensed with acetone (from the bisulfite compound) in the usual way, giving 24.5 gm. of a pale yellow syrup which was dissolved in dry ether and a trace of insoluble material filtered off. The filtrate was evaporated to dryness and the product fractionally distilled in a high vacuum. The fractions obtained are described in Table VI.

TABLE VII

Hydrolysis of "Acetone Ribulose" Fractions (0.2 N Oxalic Acid Solution)

Fraction No.	$[\alpha]_D^{25}$ (initial)	$[\alpha]_D^{25}$ (after 8 hrs. at 65°)
	<i>degrees</i>	<i>degrees</i>
1	+81	+25
2	+71	+29
3	+51	+39
4	+22	+48
5	+16	+37
6	+11	+25

Each of the first five fractions had a refractive index and elementary composition showing it to be largely diacetone pentose, whereas Fraction 6 was largely monoacetone pentose. Fraction 4 crystallized to a considerable extent on nucleation with a trace of crystalline diacetone *l*-arabinose.

The composition of the different fractions was now investigated by hydrolyzing samples with 0.2 N oxalic acid at 65° (see Table VII). In this way it was shown that, passing from Fraction 1 to Fraction 4, the proportion of diacetone *l*-ribulose diminished while the proportion of diacetone *l*-arabinose increased to a maximum; then the proportion of diacetone *l*-arabinose decreased and Fraction 6 was a fairly pure specimen of monoacetone *l*-ribose. It is possible that the anhydromonoacetone *l*-ribose was in Fraction 4, judging from the elementary composition and the hydrolysis data.

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Examination of Monoacetone Fraction

1 gm. of material from Fraction 6 was hydrolyzed with 0.2 N oxalic acid solution exactly as described for the hydrolysis of monoacetone xylulose and the free sugar isolated as a pale yellow syrup. Weight 0.7 gm. This was condensed with *p*-bromophenylhydrazine (0.9 gm.) in absolute methyl alcohol and the product isolated as described for the corresponding lyxose derivative. A first small crop of crystals proved to be *l*-arabinose *p*-bromophenylhydrazone having a melting point of 165° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.19^\circ \times 100}{2 \times 0.884} = +10.7^\circ \text{ (in absolute ethyl alcohol)}$$

A second crop of crystalline material had

$$[\alpha]_D^{25} = \frac{+0.34^\circ \times 100}{2 \times 1.950} = +16.2^\circ \text{ (in dry pyridine, 5 minutes after dissolution)}$$

and was therefore almost pure *l*-ribose *p*-bromophenylhydrazone.

d-Ribose *p*-Bromophenylhydrazone—Recrystallized from water, *d*-ribose *p*-bromophenylhydrazone had a melting point of 164–165° when heated at a normal rate. Absolute ethyl alcohol is not an appropriate solvent for determination of its specific rotation, since to obtain a 1 per cent solution it is necessary to heat the hydrazone with the alcohol. Such a solution then showed

$$[\alpha]_D^{25} = \frac{+0.23^\circ \times 100}{2 \times 1.120} = +10.3^\circ \text{ (in absolute ethyl alcohol)}$$

It was readily soluble in dry pyridine, the solution having

$$[\alpha]_D^{25} = \frac{-0.39^\circ \times 100}{2 \times 1.008} = -19.3^\circ \text{ (5 minutes after dissolution)}$$

changing to -6.0° (20 hours), $+1.0^\circ$ (48.5 hours), and $+2.0^\circ$ (67.5 hours).

l-Ribose *p*-Bromophenylhydrazone—An authentic sample of *l*-ribose *p*-bromophenylhydrazone,²⁴ having a melting point of 164–165°, had the following specific rotation in dry pyridine.

²⁴ Kindly presented to us by Dr. F. L. Humoller.

$$[\alpha]_D^{20} = \frac{+0.38^\circ \times 100}{2 \times 0.866} = +21.9^\circ \text{ (5 minutes after dissolution)}$$

Examination of Diacetone Fraction

Isolation of Crystalline Diacetone l-Ribulose—Five portions (75 gm. each) of *l*-arabinose, treated with pyridine in the usual way, gave a total of some 100 gm. of crude ribulose, which was condensed with acetone and the product fractionated to give 81 gm. of diacetone pentose fraction. This was redistilled in order to remove the major portion of the diacetone arabinose.

The crude diacetone ribulose so obtained (53 gm., having $n_D^{25} = 1.4445$ and $[\alpha]_D^{28} = +68.3^\circ$ (in acetone)) was fractionated under a high vacuum, giving a main fraction (35 gm.), boiling at 55° at 0.1 mm. (bath temperature, 59°), of a very mobile liquid which was sufficiently pure (about 90 per cent) to crystallize to a considerable extent after having been cooled to a very thick oil (in a bath of solid carbon dioxide-chloroform) and then allowed to stand in the refrigerator at $+2^\circ$. A sample of the material had $n_D^{25} = 1.4422$ and $[\alpha]_D^{27} = +94.6^\circ$ (in acetone).

For final purification a portion of the crystalline material was freed from adhering syrup by filtering through a sintered glass funnel cooled in ice-alcohol. The dry crystals so obtained had a melting point of $+5^\circ$ and

$$[\alpha]_D^{27} = \frac{+2.27^\circ \times 100}{2 \times 1.076} = +105.5^\circ \text{ (in acetone)}$$

A portion (6 gm.) of impure diacetone ribulose having $[\alpha]_D^{27} = +94.6^\circ$ (in acetone) was hydrolyzed with 0.2 N oxalic acid in the usual manner (the acetone compound dissolving slowly on shaking at 65°), giving 3.8 gm. of syrupy sugar from which a small amount of *l*-arabinose crystallized on stirring with a little absolute ethyl alcohol. The residual syrupy *l*-ribulose had, after drying, the following specific rotation.

$$[\alpha]_D^{27} = \frac{+0.48^\circ \times 100}{2 \times 1.442} = +16.6^\circ \text{ (final in water)}$$

THE EFFECT OF DINITROPHENOL ON THE METABOLISM OF FROG MUSCLE

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The marked effect of nitrophenols in accelerating the metabolism of animals (Heymans and Bouckeart, 1928; Magne, Mayer, and Plantefol, 1931, 1932; van Utyvanck, 1931; von Euler, 1932; Cutting and Tainter, 1932) prompted a number of workers to investigate the action of these substances upon cell suspensions and upon animal tissue *in vitro*. In experiments reported briefly by us in 1933 (Ehrenfest and Ronzoni) it was found that the addition of 2,4-dinitrophenol to the fluids in which cells or tissue was suspended caused an increase in the rate of oxygen absorption with various tissues of the frog (particularly marked in the case of muscle) and with yeast in the presence of glucose. These and similar observations reported about the same time by Field and coworkers (1933, 1934), Dodds and Greville (1934), and more recently by DeMeio and Barron (1934) and Krah1 and Clowes (1935) indicated that the increased rate of metabolism of animals treated with nitrophenols is due to a direct and somewhat general action of these substances as stimulants of tissue oxidation.

It seemed probable that an analysis of the mechanism of this action of 2,4-dinitrophenol on isolated cells and tissues would contribute to an understanding of the fundamental factors and steps involved in tissue respiration. With the object of learning what substrates are consumed in the increased metabolism and which of the various enzymatic activation factors are affected by the stimulant, we continued our study of the effect of 2,4-dinitrophenol on the metabolism of yeast and frog muscle, using the Warburg respirometers supplemented by chemical analysis. Our experiments with yeast will be reported in a later paper. Here we present our observations on frog muscle.

With intact frog muscle at 25° and with an approximately optimum concentration of 2,4-dinitrophenol (5 mg. per liter or 2.7×10^{-5} M) the oxygen consumption is increased to the extent of 6 to 10 times that of untreated resting muscle; these high levels of respiration, comparable to maximum levels attained by stimulation or treatment with caffeine, are maintained with little decline for as long as 5 hours. In the absence of oxygen 2,4-dinitrophenol produces an equally marked effect; the rate of lactic acid formation is increased 6 to 8 times. Both the lactic acid formed and the oxygen consumed are substantially accounted for by the glycogen lost. As in untreated muscles, no lactic acid accumulates with 2,4-dinitrophenol when the oxygen tension is sufficiently high to eliminate diffusion as the limiting factor.¹

In iodoacetate-poisoned muscles the acceleration in oxygen consumption caused by 2,4-dinitrophenol is slight and lasts only a short time. With the suppression of lactate formation in the absence of added substrate the effect of 2,4-dinitrophenol is abolished. If lactate is added, the increase in O₂ consumption on addition of 2,4-dinitrophenol is more marked, approaching the values for muscles not treated with iodoacetate. The high rate, however, is not maintained, and the O₂ consumption falls off in either case as the rigor becomes complete.

Phosphocreatine hydrolysis is accelerated by 2,4-dinitrophenol both aerobically and anaerobically and is more rapid in iodoacetate-treated muscles than in unpoisoned muscles.

The chemical changes occurring in muscle under the action of 2,4-dinitrophenol differ in one respect from those occurring in normal active muscle. During activity Cori and Cori (1932-33) have shown a marked accumulation of hexosemonophosphate. In 2,4-dinitrophenol-treated muscle there are only insignificant changes in this ester, a fact also noted by Cori and Cori (1935).

In neither resting nor active normal muscle is there a significant accumulation of intermediary products between glycogen and lactic acid unless hexosemonophosphate may be so considered. The limiting reaction of this chain must therefore either precede

¹ The results reported (Ehrenfest and Ronzoni, 1933) showing aerobic lactic acid production were due to the fact that O₂ diffusion was the limiting factor. Using thin sartorius muscles in an atmosphere of O₂ eliminates completely aerobic lactic acid formation.

or be the first reaction involving glycogen itself. Since 2,4-dinitrophenol does not increase either oxidation or lactic acid formation above that of active muscle, it is a sufficient explanation of its action to infer that it releases the limiting reaction to a rate at which it proceeds in active muscles. Oxidation of the mobilized carbohydrate then proceeds at a maximum rate, or in the absence of the normal mechanism of glycogen breakdown, as in iodoacetate-treated muscle, lactic acid may serve as substrate.

Procedure and Methods

After careful dissection and weighing, the muscles of frogs (*Rana pipiens*) were soaked for 30 minutes in cold oxygenated Ringer's solution to allow recovery. Thereafter the muscles were transferred to flasks of the Warburg respiration apparatus. For measuring oxygen consumption the flasks contained 2 cc. of phosphate-Ringer's solution of the desired pH, with NaOH in the inset to absorb CO₂. The apparatus was filled with oxygen. For the anaerobic experiments the flasks contained 2 cc. of bicarbonate-Ringer's solution and were filled with a mixture of CO₂ in N₂ to give the desired pH on its equilibration with the solution. The 2,4-dinitrophenol solution (0.2 cc.) was placed in the side arm of the vessel and added to the muscle after gaseous equilibrium was established. The water bath was maintained at 25°. The rate of oxygen consumption is expressed in terms of c.mm. of O₂ per gm. of muscle (wet weight) per hour.

After the period of manometric measurement the muscles were removed from the flasks, immediately frozen with CO₂ snow, and ground in ice-cold 5 per cent trichloroacetic acid, to which the suspension fluid was added. The filtrate was analyzed for phosphate fractions by the method of Fiske and Subbarow (1929) and for lactic acid by the microprocedure of Wendel (1933).² For glycogen the Good, Kramer, and Somogyi (1933) modification of Pflüger's method was used. Opposite muscles were used as controls in all experiments. As a basis for interpretation of results of the chemical analyses a series of analyses was performed on

² Some samples of trichloroacetic acid treated with copper-lime preliminary to lactic acid determination yield a high blank, which, when corrected for, does not interfere with the determination of known quantities of lactic acid.

opposite muscles treated alike. The order of variations found by separate analysis of pairs of opposite muscles is illustrated by the data of Table I.

Oxygen Consumption—The optimum concentration of 2,4-dinitrophenol for the increase of the O_2 consumption of frog muscle under conditions used in these experiments is between 5 and 7.5 mg. per liter. A concentration of 10 mg. per liter caused less acceleration than 5 mg.; higher concentrations were not investigated. A concentration of 5 mg. per liter was adopted for use throughout these experiments. With this concentration of 2,4-dinitrophenol the rate of O_2 consumption varied with different

TABLE I

Results (in Mg. per 100 Gm. of Muscle) to Show Agreement of Values for Opposite Muscles, Both Treated Alike

Lactic acid		Glycogen as glucose		Phosphocreatine as P	
10	12	2231	2265	70	71
11	8	2840	2814	69	70
12	13	1291	1321	73	70
9	7	1321	1360	75	72
520*	535*	375†	365†	48†	49†
600*	595*	410†	439†	51†	54†
582*	593*	590†	598†	47†	49†
560*	589*	910†	860†		

* Lactic acid after 180 minutes in N_2 + 2,4-dinitrophenol.

† Summer frogs. All other determinations made on winter frogs.

‡ Iodoacetate-treated controls; 30 minutes in O_2 , 10 minutes in N_2 .

muscles between 500 and 750 c.mm. per gm. of muscle per hour (Fig. 1, a), which is 6 to 10 times the rate for untreated resting muscle. The high rate was maintained after the first 20 minutes for approximately 3 hours, after which there was generally a decline, so that after 5 hours the rate was 4 to 6 times that of normal muscle.

The variations in O_2 consumption of individual muscles may be related to the thickness of the muscles used and to consequent differences in the adequacy of the oxygen supply in the interior of the muscle. This is indicated by the results shown in Table II. Opposite muscles both treated with 2,4-dinitrophenol were placed under identical conditions with the exception that one

was placed in O₂ and the other in air. After the oxygen consumption had been measured for 2 hours, the muscles were analyzed for lactic acid. It will be noted that even with thin sartorius muscles the O₂ consumed is less in air than in oxygen, and that in air but not in O₂ lactic acid accumulates. With thicker muscles a small amount of lactic acid accumulated even in oxygen. Inadequate O₂ supply does not account for all variations, since in muscles in which no lactic acid accumulation could be demonstrated the O₂ consumption varied between 500 and 750 c.mm. per gm. per hour.

TABLE II

O₂ Consumption and Lactic Acid Accumulation in Air and Oxygen during 2 Hours (per Gm. of Muscle)

Muscle	O ₂ consumption		Lactic acid	
	In O ₂	In air	In O ₂	In air
	c.mm.	c.mm.	mg.	mg.
Sartorius.....	990	820	0.09	0.88
“.....	960	800	0.04	0.83
“.....	1150	880	0.11	1.55
“.....	1350	950	0.04	1.64
Ilioibularis.....	1200	650	0.28	1.91
“.....	1280	660	0.42	1.79
Semimembranosus.....	950	430	0.54	1.61

The marked influence of pH on the stimulating action of 2,4-dinitrophenol on yeast (Field, Martin, and Field, 1933, 1934) is not observed in muscle. The rate of O₂ consumption is increased at all pH values tested from 5.8 to 8.5, within which range the rate varies no more than does that of different muscles at pH 7.5. The above investigators have put considerable stress on their observations on yeast that the activity of the dinitrophenol is proportional to the concentration of the non-dissociated acid residue, indicating that this form alone is active. This relation, which we confirm for yeast, does not hold for muscle.

The effect of 2,4-dinitrophenol on muscle is apparently reversible, since it may be partially removed by washing the muscle. In two experiments shown in Fig. 1, *a* the muscles were removed after 100 minutes treatment with 2,4-dinitrophenol, washed with

Ringer's solution for 30 minutes, and returned to the Warburg vessel. The subsequent oxygen consumption was reduced from a value of 10 times to 3 or 4 times the normal rate.

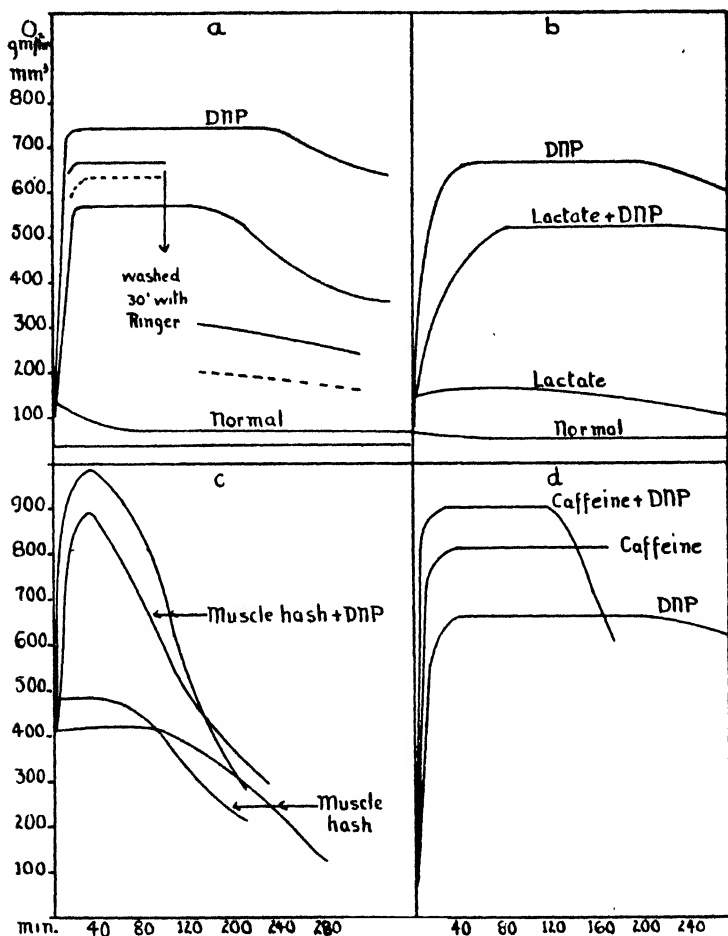


FIG. 1. The effect of 2,4-dinitrophenol on the oxygen consumption of muscle under various conditions.

A comparison of the accelerating effect due to added lactate and that due to 2,4-dinitrophenol is shown in Fig. 1, b. The addition of 200 mg. per cent of *D*-lactate as the sodium salt approximately doubles the rate of oxygen consumption in contrast to an

increase of from 8 to 10 times on the addition of dinitrophenol. With caffeine (50 mg. per cent), on the other hand, the rate of oxygen consumption was increased to a value somewhat above that of the dinitrophenol-treated muscle. This high rate caused by caffeine was only slightly further accelerated by addition of 2,4-dinitrophenol (Fig. 1, *d*).

With hashed muscle (Fig. 1, *c*) the rate of oxygen consumption is about 5 times that of uninjured muscle. This rate is further accelerated by the addition of 2,4-dinitrophenol, the maximum value reached being somewhat higher than that for intact muscle

TABLE III

Glycogen Loss in Relation to O₂ Consumption (per Gm. of Muscle) Due to 2,4-Dinitrophenol

	Glycogen as glucose	O ₂ consumption	O ₂ accounted for by glycogen
	<i>mg.</i>	<i>c.mm.</i>	<i>per cent</i>
Winter frogs	1.36	1100	92
	1.51	1420	79
	2.20	1920	85
	2.42	1953	92
	2.34	2005	87
	2.36	1931	91
Summer frogs	1.42	1037	103
	1.62	1266	95
	1.94	1520	95
	1.62	1550	78
	1.42	1310	81
	1.22	920	99
Average.....	90

similarly treated. In untreated hashed muscle lactate was formed aerobically (154 to 180 mg. per cent in 3 hours), indicating that the inhibition of glycolysis by respiration was partly abolished. That the rate of oxygen diffusion is not the limiting factor here is shown by the fact that in similar hash, treated with dinitrophenol, twice the rate of O₂ consumption was supported by the same oxygen tension. In spite of this increase in oxygen consumed on the addition of 2,4-dinitrophenol there was a further increase in aerobic glycolysis to values about double those found in untreated hash (342 to 382 mg. per cent in 3 hours).

Substrate Oxidized—In those muscles treated with 2,4-dinitrophenol and under conditions in which no lactic acid or hexosephosphate accumulated in oxygen, the decrease in glycogen accounts for from 79 to 103 per cent of the extra oxygen consumption, assuming complete oxidation of equivalent glucose to CO_2 (Table III). In a first set of experiments on winter frogs, owing to the high glycogen content and consequent greater absolute error in determination which in some cases amounted to 50 per cent of the total glycogen lost, the results show considerable variation, but an average of ten experiments gives a value accounting for 89 per cent of the total oxygen consumption. When the experiments are repeated on summer frogs with glycogen contents of around 0.5 per cent, the results are more consistent, the glycogen loss accounting for 90 per cent of the oxygen consumption. The absolute variation in initial glycogen content in these muscles was never greater than 0.15 mg. per gm. of muscle, while the total glycogen lost in these muscles was of the order of 1.5 mg. The maximum error due to variations in the initial glycogen content of opposite muscles is 10 per cent of the total glycogen lost in the experimental period. It may, therefore, be inferred that the principal substance oxidized at an increased rate under the influence of 2,4-dinitrophenol is a derivative of glycogen.

Influence of Iodoacetate—Since it appears that the main fuel oxidized under the influence of dinitrophenol in these muscles is carbohydrate, it was of interest to learn whether inhibition of lactate production by monoiodoacetate would also inhibit the acceleration of oxygen consumption.³ The results of experiments to test this question are illustrated in Fig. 2. Their interpretation requires the following analysis. As indicated in Fig. 2, *a* the oxygen consumption of an iodoacetate-treated muscle (without dinitrophenol) is approximately stationary at about the normal level or slightly below for about 100 minutes, after which time it rises with the gradual onset of rigor and later falls as the muscle dies. The effect of adding dinitrophenol to the iodoacetate-treated muscle depends upon when it is added. If added after 100 minutes, when the muscle is in or approaching rigor (Fig.

³ Muscles were treated for 30 minutes with 1:20,000 monoiodoacetate in oxygen before the experiment. This treatment was shown to prevent subsequent lactic acid formation and left the muscles with a phosphocreatine content of about 50 mg. per cent of P (Table I).

2, *e*), there is little or no additional increase of oxygen uptake, but a more rapid decline. If added earlier, when the muscle is

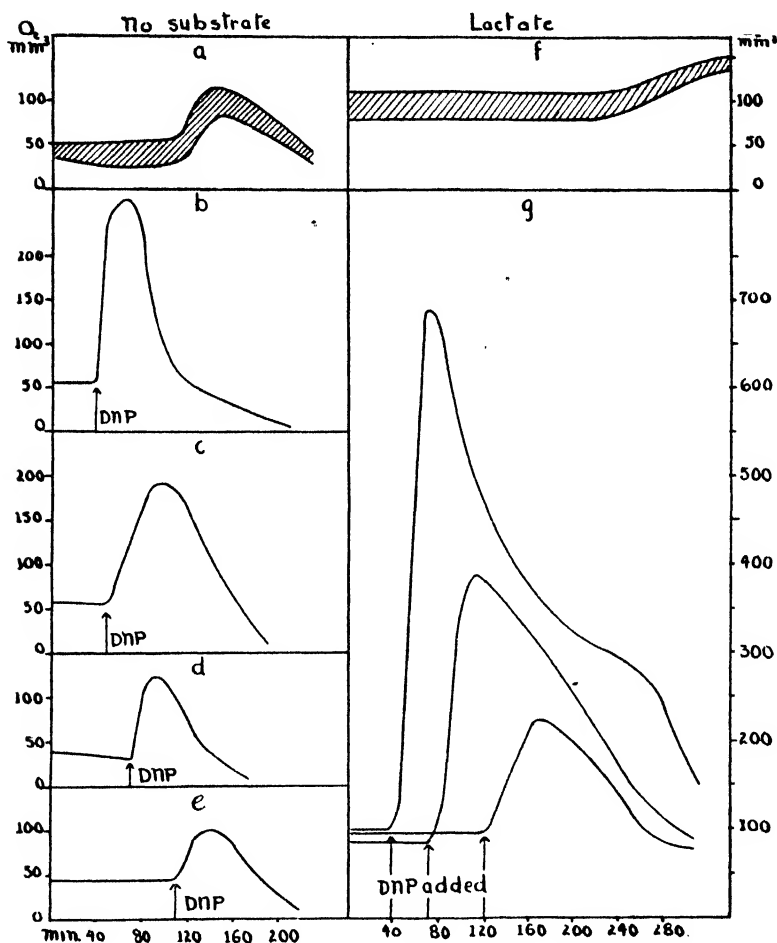


FIG. 2. The effect of 2,4-dinitrophenol on the oxygen consumption of monoiodoacetate-treated muscle with and without added lactate. *a*, without added dinitrophenol; *b* to *e*, dinitrophenol added at varying intervals after iodoacetate; *f*, without dinitrophenol; *g*, dinitrophenol added at varying intervals after iodoacetate.

less injured by iodoacetate, there is an immediate initial response but also a more rapid onset of rigor (Fig. 2, *b*, *c*, *d*). The re-

sponse is greater, the earlier the dinitrophenol is added. So long as the muscle is functionally intact, dinitrophenol causes an increase of oxygen uptake, but in the absence of reactions leading to lactate (prevented by iodoacetate), the stimulus of 2,4-dinitrophenol causes more rapid deterioration, as indicated by prompt appearance of rigor and the fall in oxygen consumption.

The addition of lactate to iodoacetate-treated muscle increases the rate of oxygen consumption and considerably delays the onset of rigor and the coincident temporary further rise of oxygen uptake (Fig. 2, *f*). This protection afforded by lactate to iodoacetate-treated muscles is accompanied by a partial restoration of the ability to maintain the normal phosphocreatine content, and hence the breakdown of adenosinetriphosphate is delayed. Mawson (1933) explains this delayed action of iodoacetate under aerobic conditions as being due to a more adequate resynthesis of phosphocreatine, made possible by the high rate of oxidation provided by the lactate.

When 2,4-dinitrophenol is added to these lactate-protected muscles (Fig. 2, *g*) there is an *initial* increase of oxygen consumption about as great (if added early) as with normal muscles. But the high level is not maintained as it is with normal muscles; the rate slowly falls and after about 5 hours has approached normal levels. The results may be summarized by saying that the addition of lactate protects the muscle for a time from the poisonous effects of iodoacetate. 2,4-Dinitrophenol exerts upon these protected muscles a more marked effect, a greater increase of oxygen uptake, but the stimulant (or the response) is also injurious, and rigor more rapidly sets in, followed by a decline in oxygen consumption. What the material is which was oxidized under the stimulus of dinitrophenol in the iodoacetate-treated muscles *without* lactate we cannot say. The accelerated oxygen consumption caused by dinitrophenol in the iodoacetate-treated muscles with added lactate was accompanied by a disappearance of lactate, slightly in excess of that accounted for, assuming complete oxidation.

The facts noted above suggest that 2,4-dinitrophenol causes an acceleration in the enzymatic activation of carbohydrate, leading either to the formation of lactic acid or to its oxidation or both as in the case of caffeine poisoning or injury due to hashing. If such

a mechanism accounts for the acceleration in oxidation, there should be some correspondence between the anaerobic formation of lactic acid and the accelerated rate of oxidation.

Effect of pH on Anaerobic Lactate Production under 2,4-Dinitrophenol—Since it was shown that lowering the pH of the surrounding solution to 6 prevents the anaerobic formation of lactic acid (Kerly and Ronzoni, 1933), this method of inhibition was also tried with dinitrophenol. The data are shown in Table IV.

While in normal muscles at pH 6 in bicarbonate-CO₂ buffer, lactic acid production is almost completely inhibited for 3 hours, when dinitrophenol is added to similar muscles the inhibition is incomplete. In a previous paper we suggested that the resumption of lactic acid production after 3 hours was probably due to liberation of a difficultly diffusible base within the muscle, pre-

TABLE IV

Effect of pH on Anaerobic Production of Lactic Acid in Presence of 2,4-Dinitrophenol (in Mg. Per Cent) in 180 Minutes

pH.....	5.6	6.1	6.5	7.4	8.5	
Phosphate buffer	83	135	384	427	581	2,4-Dinitrophenol
	0	10	22	68	103	Normal
Bicarbonate-CO ₂		56		583	605	2,4-Dinitrophenol
buffer		0		65	100	Normal

sumably from the hydrolysis of phosphocreatine, which makes the pH of the interior more alkaline than the exterior. By similar reasoning an increase in the rate of phosphocreatine hydrolysis would be expected here. That this is the case will be shown later. It is impossible to inhibit completely the production of lactic acid in the presence of 2,4-dinitrophenol by a phosphate buffer of pH 5.6 to 6 or in bicarbonate-CO₂ buffer at pH 6.1; therefore, lactic acid may not be ruled out as the substance oxidized. The data in Table IV show that at pH 7.4 or 8.5 the lactic acid production is greatly accelerated by dinitrophenol. Fig. 3 represents the rise of lactic acid with time, under anaerobic conditions. All values determined are plotted and the envelope represents the maximum variations noted. The curves for normal muscle are averages of a large series of determinations. At pH 8.5 there is a slight lag in the acceleration due to dinitrophenol; it then con-

tinues at a constant rate for a period of at least 3 hours, at which time 500 to 600 mg. of lactic have been produced per 100 gm. of muscle. At pH 7.4, on the other hand, the rate of lactic acid production begins to fall off in about 2 hours when 400 to 450 mg. of lactic acid have accumulated. This has reduced the pH of the surrounding medium to between 6.0 and 6.1, which accounts

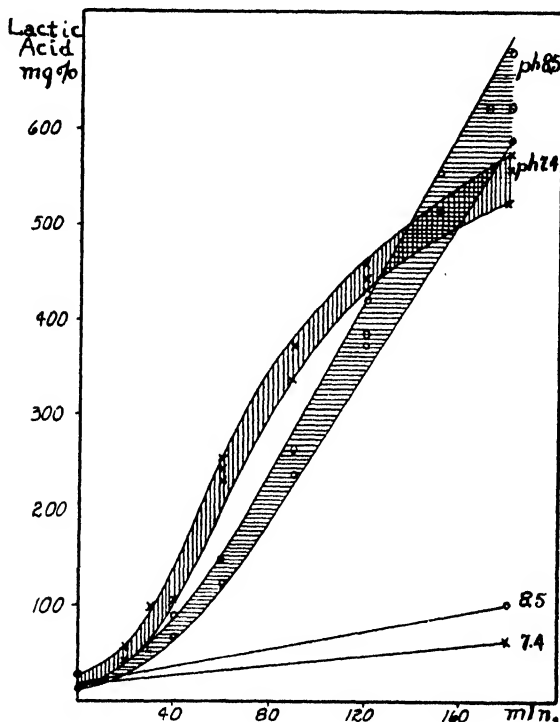


FIG. 3. The increase in anaerobic lactic acid production in muscle on the addition of 2,4-dinitrophenol.

for the falling off in the rate as shown by the fact that if at this time the bicarbonate is renewed the normal rate continues. In muscles with high initial glycogen content lactic acid production ceases before the glycogen is completely used, after the production of 1 to 1.2 per cent of lactic acid, even when the bicarbonate is renewed every 30 minutes. The final failure coincides with the complete breakdown of adenosinetriphosphate, the coferment

of lactic acid production. This is in contrast to normal muscles which in the presence of sufficient buffer are capable of converting their entire glycogen content into lactic acid under anaerobic conditions.

2,4-Dinitrophenol induces no measurable acceleration in the lactic acid production of muscle *extract* in which the rate is already maximum. The acceleration noted in muscle would, therefore, appear to consist in the removal or destruction of those factors which in normal muscle control the rate of lactate formation. Phosphate esterification is not accelerated in muscle extract by 2,4-dinitrophenol, either in the presence or absence of sodium fluoride.

In hashed muscle there is a definite acceleration of lactic acid production on addition of 2,4-dinitrophenol. For example, one muscle was placed in dinitrophenol and the other of a pair was placed in Ringer's solution. After a period of 15 minutes in N_2 both were hashed and 15 minutes later extracted for analysis. The muscle hashed in Ringer's solution alone contained 101 mg. per cent, the one in dinitrophenol 167 mg. per cent. Since a muscle kept for 30 minutes in dinitrophenol contained 90 mg. per cent of lactic acid, the additive effect of dinitrophenol and hashing determined separately is 191 mg. per cent as compared with 167 mg. per cent due to the combined action. In all cases the effects on lactate production of 2,4-dinitrophenol and the injury of hashing are supplementary, but the combined effect is less than the sum of the two determined on different muscles. The acceleration, in per cent of the already high value of hashed muscle, is less than that observed for intact muscles, but the rate of production is somewhat greater than in normal muscles treated with 2,4-dinitrophenol. The total lactate production reaches the same value in normal muscle as in hash if sufficient time is allowed. In hashed muscle treated with dinitrophenol 644 mg. per cent of lactic acid were produced in 2 hours, while in intact muscle, also treated, 3 to 4 hours are required to produce this quantity.

Effect of Stimulation on Lactic Acid Production—A comparison has been made (Table V) between the effect of stimulation on normal and 2,4-dinitrophenol-treated muscles. The excess lactic acid due to stimulation is of the same order in treated muscles as in normal muscles, except where the treatment with dinitrophenol

has been of such duration as to raise the lactate to high levels. If this has occurred, both the tension developed by the muscle and the lactic acid production due to stimulation are diminished in the muscle treated with dinitrophenol. The relation between lactic acid produced and tension has not been studied in sufficient detail to permit quantitative comparison, but it is found that whenever the muscle responds to stimulation there is an excess lactic acid production over the high level due to dinitrophenol alone.

Phosphocreatine—The work of Margaria and associates (1933) showed that a large part of the acceleration of oxygen consumption due to exercise must be referable to another mechanism than

TABLE V

Effect of Stimulation (in Mg. of Lactic Acid per 100 Gm. of Muscle) on Lactic Acid Formation in 2,4-Dinitrophenol-Treated Muscles; 10 Seconds Tetanus

Time in dinitro- phenol	Treated muscle			Untreated muscle		
	Unstimu- lated	Stimulated	Increase	Unstimu- lated	Stimulated	Increase
<i>min.</i>						
20	43	198	155	20	189	169
30	84	257	173	28	195	167
50	195	327	132	40	182	142
50	189	325	136	50	199	149
70	245	303	58	55	194	139
90	333	343	10	60	190	130

the removal of lactic acid. The oxidative resynthesis of phosphocreatine is well established, and we have shown an acceleration of oxygen consumption in muscles paralleling the resynthesis of phosphocreatine in the absence of lactic acid removal (Ronzoni and Kerly, 1933). Hegnauer, Fenn, and Cobb (1933-34) also have found an acceleration of oxygen consumption in muscle due to excess potassium in the absence of lactate production and coincident with an increase in phosphocreatine hydrolysis. It was therefore of interest to determine the effect of 2,4-dinitrophenol on the behavior of phosphocreatine. The curves in Fig. 4, *a* show the acceleration in breakdown of phosphocreatine when dinitrophenol is added to aerobic and anaerobic muscles at pH 7.4. If the final concentration of phosphocreatine is regarded as the

balance between hydrolysis and resynthesis, it appears that 2,4-dinitrophenol causes either an acceleration of its hydrolysis or an inhibition of its resynthesis. Experiments under anaerobic conditions with iodoacetate-treated muscles, in which the resynthesis of phosphocreatine by the lactic acid mechanism is eliminated, show that dinitrophenol accelerates the hydrolysis (Fig. 4, *b*). The difference between the two curves can perhaps be considered as the amount of phosphocreatine rebuilt owing to the lactic acid mechanism alone. Since the rate of phosphocreatine hydrolysis in iodoacetate-poisoned muscle is approximately doubled and the

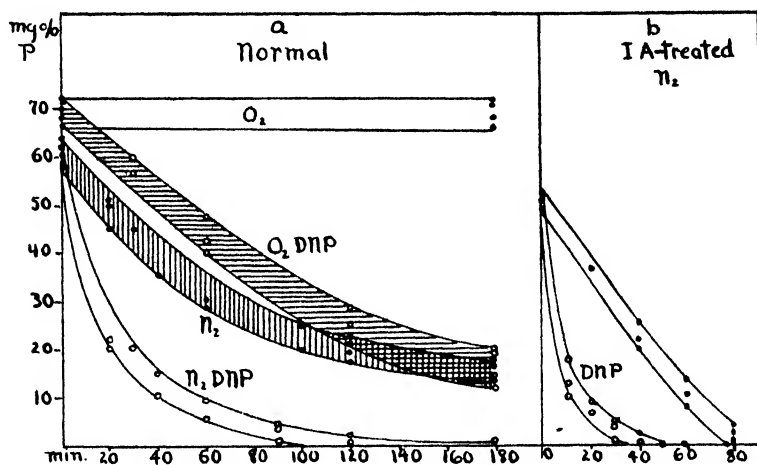


FIG. 4. Changes in phosphocreatine content of muscle as influenced by the addition of 2,4-dinitrophenol. *a*, normal muscle; *b*, treated with monoiodoacetate.

rate of lactic acid formation in unpoisoned muscle is increased 6 to 8 times, it would appear to follow that the coupling of anaerobic resynthesis of phosphocreatine with lactate formation is less effective under the influence of dinitrophenol.

When ample oxygen is available to normal muscle, there is no loss of phosphocreatine. However, if a fully oxygenated muscle is treated with 2,4-dinitrophenol, the rate at which phosphocreatine is resynthesized is overbalanced by the rate of its hydrolysis and in spite of an acceleration of 6 to 10 times in the rate of oxygen consumption phosphocreatine tends to diminish in the muscle.

There is, however, some resynthesis or inhibition of hydrolysis by oxygen shown by the fact that the decrease in phosphocreatine is greater in the absence of oxygen. Here again if we consider the degree of acceleration of the two processes separately, the oxygen consumption due to 2,4-dinitrophenol is less effective in preventing the hydrolysis of phosphocreatine than in normal muscles.

If we compare the magnitude of the acceleration in lactic acid formation with the increase in the rate of hydrolysis of phosphocreatine caused by dinitrophenol, we find that in anaerobic muscles lactic acid formation is accelerated between 7 and 8 times the normal rate and hydrolysis of phosphocreatine is only about doubled. The increase in lactic acid production compares favorably with the increase in oxygen consumption. On the other hand, the concentration of lactic acid in aerobic muscles must at any time be very low, and by its concentration alone cannot account for the observed acceleration of oxygen consumption.

The cycle of chemical changes in muscle associated with activity involves as one of its critical features the exchange of phosphate, which passes through a cycle of compounds starting with adenosinetriphosphate. At one point in this cycle energy is yielded for muscle contraction (or for restoration of the contractile mechanism); at another point energy is gained, for the restoration of the normal phosphate equilibrium, from sugar breakdown. The striking feature of this cycle is that in normal intact muscle it runs down spontaneously only very slowly to the complete utilization of the energy-yielding carbohydrate. The various stages appear to be in an equilibrium which is maintained at a low energy cost, derived from carbohydrate splitting. The energy so derived is capable of completely maintaining this equilibrium oxidatively, but anaerobically the cycle runs down slowly and stops with the final complete hydrolysis of phosphocreatine and adenosinetriphosphate. The critical link in restitution appears, then, to be the transfer of energy and phosphate from carbohydrate esters to creatine in the resynthesis of phosphocreatine through adenosinetriphosphate as mediator of phosphate (Parnas, Ostern, and Mann, 1935; Needham and van Heyningen, 1935; Lohmann, 1935). When phosphopyruvic acid is dephosphorylated and converted into lactic acid anaerobically, while the phosphate liberated is sufficient, restoration is incomplete in intact muscle and

inorganic phosphate appears. With the oxidative disposal of the intermediate the restoration is complete.

The various stages of the cycle are then maintained in normal equilibrium at a very low energy cost until excitation of the muscle disposes of the energy at one point, presumably the hydrolysis of adenosinetriphosphate which is immediately restored from the phosphate reserve, phosphocreatine, whereupon the cycle runs until this energy and phosphate are restored. Here again the anaerobic restoration is not quite complete, while oxidatively the equilibrium is restored if sufficient time is allowed.

If at any one point in this cycle one of its reactions were inhibited, all reactions beyond that would soon cease. Monoiodoacetate in preventing the dismutation of the triosephosphate prevents the restitution of phosphocreatine and finally of adenosinetriphosphate, and the cycle runs down from this point with the conversion of a part of the phosphate of phosphocreatine and of adenosinetriphosphate into carbohydrate esters, the remainder appearing as inorganic phosphate. The fact that the cycle runs down more slowly in the presence of oxygen, especially if an oxidizable substrate is added, is presumably due to oxidative resynthesis of phosphocreatine. The transference of energy to the contractile process appears to be through the hydrolysis of adenosinetriphosphate, since this must precede the formation of hexose esters as well as the hydrolysis of phosphocreatine. This, therefore, must be the point of release of the control of carbohydrate mobilization. There is normally no measurable breakdown of adenosinetriphosphate, since its restitution by phosphocreatine hydrolysis is rapid and complete and the hydrolysis of phosphocreatine becomes the measurable evidence of what has occurred. Muscle excitation and 2,4-dinitrophenol both accelerate the breakdown of phosphocreatine and presumably, therefore, the rate of hydrolysis of adenosinetriphosphate, which is in turn accompanied by a mobilization of carbohydrate. The normal phosphate equilibrium as well as the energy of the system has been disestablished and the cycle runs until the equilibrium has been reestablished after muscle excitation. Failing this, under the influence of dinitrophenol the reaction proceeds not to the complete breakdown of carbohydrate, but, owing to the imperfect resynthesis of phosphocreatine, to the complete breakdown of phosphocreatine and adenosinetriphosphate, which brings it to a stop.

Since the rate of oxygen consumed under the influence of dinitrophenol does not exceed that which may be produced by other means (caffeine treatment or excitation), it is reasonable to assume that the stimulating factor is the same in either case. The acceleration in oxygen consumption and the acceleration in anaerobic lactic acid formation bear a direct relationship in 2,4-dinitrophenol-treated muscles, and it is again reasonable to assume that the mechanism of mobilization of carbohydrate is the same in either case. The anaerobic production of lactic acid due to dinitrophenol approaches the maximum owing to stimulation, but does not prevent further response due to excitation. In muscle extract where the entire mechanism is released, dinitrophenol has no accelerating effect on lactic acid production or on hexosephosphate accumulation. This marked correspondence between the acceleration of anaerobic processes on the one hand and oxygen consumption on the other points to a generally stimulated enzymatic activity caused by the release of the normal controlling factors as the mechanism of the action of 2,4-dinitrophenol.

SUMMARY

1. 2,4-Dinitrophenol at optimum concentrations accelerates the oxygen consumption of green frog muscle 6 to 10 times. The excess oxygen consumed above the normal is substantially accounted for by the glycogen lost. In iodoacetate-treated muscles the acceleration due to 2,4-dinitrophenol is small. The addition of lactate partially restores the accelerating effect of dinitrophenol on oxygen consumption.

2. The anaerobic lactic acid production is accelerated 7 to 8 times the normal rate, and, if adequately buffered, continues until the complete breakdown of phosphocreatine and adenosinetriphosphate. In muscles with high glycogen content (2.0 to 2.5 gm. per 100 gm.) the reaction ceases before the carbohydrate is completely utilized.

3. Oxygen prevents lactic acid accumulation, but it cannot prevent completely the breakdown of phosphocreatine and in the long run of adenosinetriphosphate.

4. 2,4-Dinitrophenol accelerates the rate of phosphocreatine hydrolysis above that which can be restored by the accelerated

rate of oxidation. The rate of hydrolysis is shown to be approximately doubled. Since the O_2 consumption and lactic acid production are accelerated 6 to 10 times the normal, resynthesis by these two mechanisms must be interfered with by dinitrophenol.

5. Since 2,4-dinitrophenol accelerates phosphocreatine hydrolysis, which takes place only in the presence of adenylic acid, it must also accelerate the hydrolysis of adenosinetriphosphate.

6. In muscle extract, freeing the enzymes, their activators, and substrate from cell structure releases the controlling factors of carbohydrate breakdown and dinitrophenol is now without influence on the rate of lactic acid production or of phosphate esterification.

7. 2,4-Dinitrophenol, therefore, appears to act by allowing the maximum mobilization or activation of carbohydrate, which leads to an acceleration of oxygen consumption or anaerobically to accelerated lactic acid formation.

8. Normally the function of the hydrolysis of adenosinetriphosphate appears to be the restoration of the contractile mechanism. Since with dinitrophenol there is no such restoration necessary, the energy of the accelerated activity is thus wasted.

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REMARKS ON THE PAPER BY TENDELOO ON A NEW AND EASY METHOD FOR THE POTENTIOMETRIC DETERMINATION OF CALCIUM CONCENTRATIONS IN SOLUTIONS

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In a recent publication,¹ Tendeloo has proposed an ingenious method for estimating concentrations of calcium in solution through the use of a fluoride, CaF_2 , electrode. If this electrode method were valid, it would be a useful tool in the service of biological investigation. However, examination of the results obtained by Tendeloo with this electrode on the adsorption of calcium by gelatin from calcium nitrate solutions leads to considerable doubt regarding its reliability.

Tandeloo reports that gelatin adsorbs a considerable amount of calcium from solutions more acid than the isoelectric point of gelatin, which is contrary to the current views on the physico-chemical properties of the proteins. Furthermore, the quantities of calcium which are stated to be adsorbed by the gelatin, ranging up to amounts of 0.5 and 0.74 gm. of calcium per gm. of gelatin, appear improbably large.

Because of the unusual nature of these results, it appeared desirable to subject them to test by an independent means. Accordingly, experiments on the adsorption of calcium by gelatin were carried out by ultrafiltration; solutions similar in composition to those reported in Table III of the work of Tendeloo were employed.

Eastman, electrolytically deashed gelatin was employed. This was dissolved in a calcium nitrate solution of approximately 0.5 N, a correction being applied for the moisture content of the gela-

¹ Tendeloo, H. J. C., *J. Biol. Chem.*, **113**, 333 (1936).

tin. One series of experiments was carried out at the natural pH of the gelatin and another adjusted to the pH of 4.3 with hydrochloric acid. The calcium concentrations of the original solutions and the ultrafiltrates were determined by precipitation of the calcium as oxalate and titrating with permanganate.

The results which were obtained are reported in Table I. From the data given in Table I, it is clear that at the isoelectric point, and in more acid regions of pH, gelatin adsorbs none or only a negligible amount of calcium.

The experimental results are in harmony with the current views on the physicochemical behavior of the proteins, and throw doubt on the reliability of the fluoride electrode as a means of determining calcium ion concentrations.

TABLE I

Test for Adsorption of Calcium by Gelatin from Calcium Nitrate Solutions

Gelatin concentration	pH 4.8		pH 4.3	
	Initial Ca concentration	Ultrafiltrate Ca concentration	Initial Ca concentration	Ultrafiltrate Ca concentration
<i>per cent</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>
0.5	568	569	555	561
1.0	568	575	557	560
1.5	568	577	553	563
2.0	568	578	552	555
2.5	568	570	553	550

FURTHER OBSERVATIONS ON THE CHEMICAL NATURE OF A HEMATOPOIETIC SUBSTANCE OCCURRING IN LIVER

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About a year ago two of the present authors (1) described the preparation and partial purification of a product from liver which was effective in causing blood regeneration in pernicious anemia. The method of purification which was fully described consisted essentially in the removal of much inert material with alcoholic calcium acetate, the precipitation of the active material with Reinecke acid, its regeneration from the Reineckate under special conditions, and finally its fractionation by means of salting-out with a variety of salts. The active material was also isolated as picrate and flavianate. In this communication it was stated that,

"It may at once be emphasized that the material to be described has not been crystallized and such crystalline derivatives as have been obtained are of little value in establishing strict chemical individuality; in fact, the available evidence is against the view that we are dealing with a single substance. On the other hand, relative constancy of composition, the isolation of crystalline products of hydrolysis, and the apparent fact that clinical potency is consistently associated with the compound and is absent from preparations in which it has been either removed or chemically altered, would seem to justify its claim to consideration."

The clinical potency of material prepared according to this method has been independently confirmed by Ungley, Davidson, and Wayne (2), working under the auspices of the British Medical Research Council, using a commercial product¹ prepared under

* Leverhulme Scholar of the Royal College of Physicians.

¹ Obtainable from The British Drug Houses, Ltd., London, under the name anahæmin.

the direction of Dr. F. H. Carr, and by Wilkinson (3) using material supplied by Dr. F. L. Pyman. The object of the present communication is to describe some further steps in the purification of the antipernicious anemia principle of liver, and to record certain deductions that may be drawn from the chemical data thus acquired.

It may be recalled that the product previously described gave on hydrolysis an aminohexose similar to glucosamine, and lysine, arginine, glycine, leucine, hydroxyproline, aspartic, and other dibasic acids. The presence of glucosamine was of considerable interest for a variety of reasons. Thus it was stated in our previous paper that, "The possible relationship of our substance to products of the partial digestion of gastric mucin [containing glucosamine] is obvious and is of interest in connection with the therapeutic effects of dried gastric tissue...and of concentrated gastric juice." Our suspicion that glucosamine might be an essential grouping in the liver product was strengthened by experiments which we made on the isolation of similar products from kidney and brain, both of which organs have been shown to be therapeutically active on oral administration. Thus Castle (4) reflecting the generally accepted view states that "the animal kidney is as effective a source of the active principle of liver as is that organ itself." Using chemical methods identical with those successfully employed in the case of liver, we were able to isolate peptide mixtures which unlike those from liver gave strong biuret reactions, and were completely free from glucosamine. When tested clinically, these products were found to our surprise to be totally inactive. Whether kidney and brain are sources of Castle's "intrinsic factor" remains to be determined, but by our method they do not yield significant amounts of the antipernicious anemia principle found in liver. In this connection it may be mentioned that we have also tested mucin-rich salivary glands desiccated at low temperature and found that they possess no therapeutic activity when fed to a pernicious anemia patient.

In the light of the preceding results we were encouraged to try to separate our product from liver into glucosamine-rich and glucosamine-poor fractions. By the use of methods described later we have convinced ourselves that glucosamine is not an integral part of the active principle. We have obtained preparations

surpassing in clinical activity those previously described which were completely free from glucosamine, while on the other hand the glucosamine-rich preparations showed either no clinical activity or a diminished one. Our previous suggestion that there might possibly be a relationship between glucosamine-rich gastric mucin and the active substance in liver must obviously be withdrawn. The difficulty which we experienced in securing glucosamine-free preparations from liver is not surprising in view of the extraordinarily high glucosamine content of liver proteins. Thus more than 30 years ago Neuberg and Milchner (5) showed that liver proteins contained at least 3.58 per cent of glucosamine and that on autolysis or digestion it was not set free.

The preparation of glucosamine-free preparations is based on the following considerations. Taking as starting material preparations corresponding to Product II as described in our earlier paper, we found that while neutral lead acetate gave no precipitate at all, a small but variable amount of material could be precipitated by basic lead acetate and freshly precipitated lead hydroxide (we have used Horne's basic lead acetate exclusively). The precipitated material after hydrolysis was found by Elson and Morgan's (6) colorimetric method for glucosamine estimation to contain a considerably higher percentage of aminohexose than the original material. On regenerating the material from this precipitate, it was found to be clinically inactive (Case 1). The activity of the material in the filtrate was confirmed (Case 1). By the cautious addition of ammonia to the filtrate from the preceding experiment, a small additional precipitate was obtained which also contained much aminohexose. In some cases the material so recovered showed no clinical activity, while in others it was clear that some of the active compound had been precipitated (Case 2). While much aminohexose-peptide has been removed by the preceding treatment, the filtrate still contains significant amounts of glucosamine, and a positive Molisch reaction such as is given by glucosamine peptides is invariably obtained. The next step in the purification of the clinically active material in the filtrate was based on the following observation. It was found that our substance was precipitable by uranium acetate. On the addition of uranium acetate the precipitate first separating contains extremely little glucosamine but is clinically highly active

(Case 3). The mother liquor after the initial precipitation becomes definitely acid and a further precipitate containing almost the whole of the residual peptide is obtained on the cautious addition of ammonia until approximate neutrality is reached. The material derived from the acid filtrate still contains a good deal of glucosamine and is clinically active (Case 1), though probably less so than that from the first precipitate. The regeneration of the uranium precipitates was at first accomplished by simple agitation with neutral ammonium phosphate, but later it was found more convenient and to permit more nearly quantitative results to dissolve the precipitate in 0.5 N sulfuric acid, add an excess of ammonium phosphate, and bring to pH 6 with ammonia. After filtering off the uranium phosphate, the peptide could be recovered from the filtrate either by precipitation with Reinecke salt or by salting-out with ammonium sulfate. The various fractions obtained by the above procedures were all examined for glucosamine after hydrolysis with normal hydrochloric acid for 1 to 2 hours at 100°. It should be stated that the results obtained by Elson and Morgan's method were invariably much higher than those obtainable by any copper reduction method, and while, as stated in our first paper, this extension of Elson and Morgan's method may be beset with serious quantitative errors, it at least proved invaluable for the rapid determination of maximum glucosamine values.

The following is an outline of the actual preparation of one of our glucosamine-free preparations and may be regarded as typical of the various procedures, the precise order being varied according to circumstances. It will be noted that preliminary purification with alcoholic calcium acetate is replaced by twice salting-out with saturated ammonium sulfate, a procedure that was first employed by F. H. Carr.

Liver extract powder, 1 kilo (No. 343, Eli Lilly and Company) was stirred with 2.5 liters of warm water until dissolved. Ammonium sulfate, 1400 gm., was then added by degrees with constant stirring and the mixture placed in a refrigerator overnight. The precipitate was filtered off on a Buchner funnel, washed with saturated ammonium sulfate solution, pressed out thoroughly, and sucked as dry as possible. It was then vigorously stirred with 500 cc. of distilled water so as to get as much as possible in solution and towards the end of the stirring 20 gm. of filter-cel were added, and the whole filtered with suction with a minimum

of wash water. Reprecipitation is then carried out by again saturating with ammonium sulfate, with care to avoid excess. The precipitate was filtered off, sucked dry, and suspended in 200 cc. of water and well stirred, and after a short time 500 cc. of alcohol were added by degrees and the mixture allowed to stand some hours in the refrigerator. The precipitate of ammonium sulfate and alcohol-insoluble peptides was filtered off and washed with 70 per cent alcohol. The filtrate was then concentrated under diminished pressure to remove alcohol at a temperature below 50°.

The first precipitation with basic lead acetate was now carried out with about 200 gm. of basic lead acetate dissolved in a minimum of warm water, and by cautiously adding ammonia until the mixture was quite definitely alkaline to litmus. Filtration was carried out immediately and the filtrate acidified to Congo red with sulfuric acid as soon as possible, and the lead sulfate removed by filtration. The next step involved precipitation of the active material with Reinecke acid, of which about 25 gm. were required. The carrying out of this precipitation and the subsequent decomposition of the precipitate dissolved in methyl alcohol with dimethylaniline has been fully described in our earlier paper and need not be repeated in detail. The solution of the active material was freed from methyl alcohol by concentration under reduced pressure and next precipitated with uranium acetate, of which about 150 cc. of saturated solution (10 gm.) were required. The acidity of the solution was diminished by adding 10 cc. of 1:10 aqueous ammonia, but the final reaction was maintained distinctly acid to litmus. The curdy precipitate which filtered easily was sucked off and well washed with cold water, dissolved in 0.5 N sulfuric acid, and the uranium removed as phosphate by adding ammonium phosphate (2 gm.) and ammonia to neutrality. The solution, which still contained significant amounts of combined glucosamine, was again subjected to precipitation with basic lead acetate (15 gm.), freshly prepared lead hydroxide (5 gm.), and ammonia was added as long as an additional precipitate was formed. The precipitate on decomposition with sulfuric acid was found to contain the whole of the glucosamine, while the filtrate contained none. The peptide in the filtrate was recovered by acidifying with sulfuric acid, filtering off the lead sulfate, and again precipitating with Reinecke acid (3 gm.). The Reineckate was decomposed as previously described and the solid peptide

recovered by concentration and precipitation with absolute alcohol. The yield of glucosamine-free product was 4.02 gm. The Molisch reaction was completely negative in 1:30 dilution, and on hydrolysis 100 mg. gave a doubtful trace of color with the acetylacetone reagent. The amount of combined glucosamine, if any were present, was certainly less than 0.1 per cent. It must be noted, however, that not every preparation obtained by the preceding method was equally free from combined glucosamine, for some showed from a definite trace to a maximum of a fraction of 1 per cent as determined by the acetylacetone method.

The reservations which we made regarding the purity of our previous preparations must be again emphasized, although the degree of constancy of composition is rather surprising. In view of the fact that no albumose of natural origin has ever been purified to the extent demanded by strict chemical criteria, it can hardly be expected that we should have better fortune. It is unfortunately true that none of the steps used in the preparation can in any sense be regarded as specific for our substance, but are rather in the nature of group reactions common to similar compounds. It is also disturbing to realize that on subjecting a protein such as gelatin to peptic digestion and then applying our methods to the separation of the products formed, it is found that a not inconsiderable amount of albumose, soluble in 70 per cent alcohol, appears in the final stages. The products, however, uniformly give strong biuret reactions, unlike the substance from liver. With these limitations in view, it is of interest to compare the composition of our present glucosamine-free preparations with those of our Product II described in our first paper, which we were inclined to regard with the most favor as showing the lowest amino nitrogen and the highest specific rotation.

	Product II	Glucosamine-free preparations
	<i>per cent</i>	<i>per cent</i>
Carbon.....	50.0- 51.4	46.8- 48.1
Hydrogen.....	7.0- 7.2	6.6- 6.8
Nitrogen.....	15.2- 15.4	15.9- 16.5
NH ₂ (Van Slyke).....	0.4- 0.5	0 - 0.2
NH ₂ -N after hydrolysis.....	10.6- 10.8	10.0- 10.4
Specific rotation, $[\alpha]_D^{25}$, degrees.....	-95 to -106	-112 to -133

It will be noted that the glucosamine-free preparations show a slightly lower carbon content and a slightly higher nitrogen. We believe this difference may be explained by the presence of more glycine in our new preparations. The complete or almost complete absence of amino nitrogen is noteworthy as a probable indication that the very small amount of lysine previously observed is not an essential component of the active material. The specific rotation is materially higher than previously observed. Reference must be made to the fact that the specific rotation of our substance in aqueous solution is influenced by small temperature changes to a rather surprising degree, the rotation diminishing with increase in temperature. For example, a solution which showed an angular rotation of 9.60° at 17° and 9.31° at 20° showed a rotation of 11.75° when cooled to 5° . A parallelism between high specific rotation and high clinical potency is not to be assumed. In fact, a fractionation of our earlier preparations carried out under F. H. Carr's direction, disclosed a possibly greater clinical activity of the fraction with the lower specific rotation of about -80° compared with the fraction showing a rotation of -115° (Cases 8 to 10).

Clinical results following the intramuscular injection of preparations practically free from glucosamine are furnished by Cases 2 to 7. The potency appears to be rather more than twice that previously recorded by us.

Since our substance in many particulars resembles substances collectively described as albumoses, it seemed of interest to examine the effect of a number of albumose reagents. In the first place we wish to make a correction. In our earlier paper we stated that "our substance is not precipitated by trichloroacetic acid." This is only true to a limited extent, for strong solutions are precipitated by trichloroacetic acid provided a high concentration of the latter is present. Thus, 5 per cent trichloroacetic acid precipitates almost none, 10 per cent less than half, while 20 per cent precipitates almost completely. Rufanic acid, ferrocyanic acid, and metaphosphoric acid give no precipitate, nor do copper acetate and ferric sulfate, all of them precipitants of typical albumoses. Aluminum hydroxide adsorbs it to a considerable extent, while copper hydroxide adsorbs very little. Hunter's (7) protamine reaction with albumoses is negative with clupein as

reagent. Zinc sulfate is effective as a precipitant only on complete saturation. On the other hand, uranium acetate (but not uranium nitrate), rhodanilic acid, and catecholarsenic acid are good precipitants.

Hydrolysis—The glucosamine-free peptide is readily hydrolyzed by boiling with mineral acids and, unlike the glucosamine-containing products, very little pigmentation accompanies the change. We have been able to find nothing but the usual amino acids among the products of hydrolysis. The methods used for their identification were those described in our earlier paper and need not be repeated. As already stated we have been able to eliminate glucosamine and apparently lysine as essential components, but arginine, glycine, leucine, aspartic acid, and hydroxyproline, all of which were found in our earlier preparations, have been again identified in our new preparations. The relative amounts of these amino acids approximate those previously found, with the exception that we now find 9.5 to 10.4 per cent of glycine in place of 4 to 6 in the earlier preparation. The estimation was carried out by Bergmann's method, with tripotassium chromium trioxalate as precipitant, and the results must be regarded as minimal values. A somewhat special interest attaches to the presence of glycine, for it may possibly furnish the explanation of why glycine-free casein is not a source of extrinsic factor, using Castle's terminology, and may also be connected with dietary anemias. In addition, we have obtained analytical evidence indicating that proline accompanies the hydroxyproline. We were able to identify the proline by precipitation with Bergmann's rhodanilic acid after preliminary separation as Reineckate. It should be noted that very significant amounts of proline and hydroxyproline escape the first precipitation as Reineckate and may be recovered in the form of their hydantoins on ether extraction. The analyses of these hydantoins indicated about equal parts of proline and hydroxyproline. In discussing the dicarboxylic acids in our earlier paper we referred to "indications of a dicarboxylic acid easily soluble in water and giving a very soluble copper salt precipitable by alcohol" which was obtained after the removal of aspartic and glutamic acids. We have encountered this action repeatedly in our present work. The acid which was isolated from the silver salt has a very small positive rotation ($+2.0^\circ$). On boiling with so-

dium hydroxide and diazotized sulfanilic acid, the solution which is at first colorless develops a strong cherry-red color, while with β -naphthol and sulfuric acid it gives a yellowish green fluorescence. Various preparations of the copper salt gave the following results on analysis.

Cu 26.6, 28.0, 27.3; $\text{NH}_2\cdot\text{N}$ 6.12, 6.40, 6.24

$\text{C}_6\text{H}_7\text{O}_5\text{NCu}$ requires Cu 28.3, N 6.23. The carbon analyses were also unsatisfactory, being 1 to almost 2 per cent too high. It is difficult to avoid the supposition that hydroxyglutamic acid was present, although we are conscious that the natural occurrence of this amino acid has received much skeptical comment. Larger amounts of material than we have yet had at our disposal will be necessary before we reach a final decision. One other product of hydrolysis remains to be mentioned, namely ammonia. In our early preparations the presence of glucosamine and its decomposition products made the determination of "amide" nitrogen impracticable. We find that in our present preparation about 0.5 per cent of nitrogen is in amide form. Before analysis the preparations were dissolved in a little water, magnesium oxide added, and the mixture allowed to stand over sulfuric acid for a couple of days. This precaution was taken in order to eliminate the possibility of preformed ammonium salts being mistaken for amide nitrogen.

Fractional Salting-Out with Ammonium Sulfate—The fact that ammonium sulfate, magnesium sulfate, sodium chloride, and other salts could be used for the precipitation of the active material in liver was dealt with in our earlier paper. The experiment to be reported aimed at defining a little more exactly the limits of precipitation with ammonium sulfate and also to see whether the various fractions showed marked variation. A clinically potent product (4.84 gm.) prepared as described in the earlier part of this paper was dissolved in 10 parts of water and with constant stirring brought to one-half saturation with ammonium sulfate. After standing in a cool place, the supernatant fluid was poured off and brought to two-thirds saturation with ammonium sulfate. The whole process was then repeated three times. It is noteworthy that the third one-half saturated mother liquor contained as much solid material as did the first. The mother liquors from

the two-thirds saturation were acidified with sulfuric acid (pH 2) and saturated with ammonium sulfate, but very little material had escaped precipitation. Each of the various fractions was dissolved in water so as to give approximately a 2 per cent solution and then examined in the polarimeter. The small amount of adhering ammonium sulfate was disregarded, while the concentration of the polarized solutions was based on nitrogen estimations after removal of ammonia. The material was thus fractionated into seven different fractions. The original material had $[\alpha]_D = -122^\circ$; and each of the seven fractions showed rotations within the limits of -116° and -121° . The material precipitated three times in one-half saturated ammonium sulfate amounted to 3.15 gm. (65 per cent) and had $[\alpha]_D = -121^\circ$; the material precipitated between one-half and two-thirds saturation with ammonium sulfate amounted to 1.50 gm. (33 per cent) and had $[\alpha]_D = -116^\circ$, while only 0.2 gm., $[\alpha]_D = -118^\circ$, was recovered on complete saturation. It is clear therefore from this experiment that almost all of our product is precipitated at two-thirds saturation with ammonium sulfate and most of it at one-half saturation, and also that the fractions show a surprising degree of uniformity as regards optical rotation. The clinical testing of the two main fractions, one-half and two-thirds ammonium sulfate saturation, failed to disclose significant differences (Cases 11 to 15).

Action of Alkali—In our earlier paper it was stated that "24 hours exposure to 0.5 N sodium hydroxide at room temperature completely inactivates the substance, and undoubtedly much less drastic conditions would be equally effective." It was also shown that this inactivation of clinical potency was accompanied by the racemization of certain of the amino acid groups, notably arginine, lysine, and leucine. Even before we had obtained these results, we had learned to be apprehensive as to the effect of alkali on the activity of our products and had avoided it as much as possible. Notwithstanding these fears, we have had to make use of lead salts and ammonia in order to obtain carbohydrate-free products as described in the present paper. It was therefore of some importance to determine how destructive was the action of ammonia on the purified substance. In the absence of available clinical cases we have had to resort to observing changes in optical rotation in order to detect change. Accordingly solutions were pre-

pared in 1 N ammonia and sodium hydroxide and the rotations observed at suitable intervals.

	Solution in 1 N NH ₄ OH	Solution in 1 N NaOH
	degrees	degrees
Initial rotation.....	-3.75	-3.00
After 2 hrs.....	-3.62	-2.55
“ 8 “.....	-3.55	-2.12
“ 1 day.....	-3.45	-1.64
“ 2 days.....	-3.37	-1.26
“ 3 “.....		-1.10
“ 4 “.....		-1.07

The vastly greater speed and extent of reduction in optical activity by the normal sodium hydroxide as compared with normal ammonia is obvious. It would seem probable that the short exposure to dilute ammonia which we employed in our preparation would not be productive of much chemical change.

Dialysis and Ultrafiltration—We have made a number of experiments on the dialysis and ultrafiltration of our product which may ultimately be of some value in estimating molecular size. We find that our substance dialyzes quite easily through thin sheep cecum (so called fish skin condom), much less rapidly but eventually completely through thin viscose sausage casing, while dialysis through Schleicher and Schüll parchment is extremely slow. We have tested some of the viscose dialysates and residues clinically (Cases 16 and 1). Much of the active material was removed by 18 hours dialysis. Cohn, Minot, Alles, and Salter (8) report a patient showing “an unexpectedly weak reticulocyte response when fed extract after ultrafiltration through a collodion membrane,” but drew no inferences from this observation. Through the kindness of Dr. J. H. Bauer we have been able to examine the passage of our substance through graded collodion membranes by use of the apparatus and standardized membranes described by Bauer and Hughes (9). Using a 0.5 per cent solution, we find that on filtration at 40 to 45 pounds pressure the filtrate passing through a membrane with average pore diameter of 3.78 $m\mu$ had one-third the concentration of the original solution; a membrane with average pore diameter of 3.00 $m\mu$ gave a filtrate of one-sixth

the concentration of the original solution; while a membrane with $2.5\ m\mu$ pores allowed none of the dissolved peptide to pass through it. For purposes of comparison it may be noted that egg albumin with a molecular weight generally estimated at 34,500 has a molecular size of $4.3\ m\mu$ and has a filtration end-point of $6\ m\mu$, below which the substance does not pass. Our substance passes in limited amount through a membrane of average pore size of $3\ m\mu$, indicating a molecular size of half that of egg albumin, *i.e.* $2.1\ m\mu$, and if it has the same density as most proteins, its molecular weight should be less than 5000. On the other hand, clupein sulfate, one of the simpler protamines, with an assumed molecular weight of at least 2000, under similar conditions readily passes through a membrane with average pore diameter of $2.20\ m\mu$, while our substance fails to pass through pores of $2.50\ m\mu$ average diameter. We therefore tentatively conclude that the molecular weight of our substance is considerably greater than 2000 and less than 5000 and that its molecular size approximates $2.1\ m\mu$.

It may be added as a curious fact that Congo red, which has been used in the treatment of pernicious anemia and which has a calculated molecular weight of 646 without allowing for molecular association in solution, is almost completely held back by membranes with average pores of $3.76\ m\mu$ and also by the viscose dialysis tubes used by us.

Action of Enzymes—The action of crystalline pepsin, of pancreatic juice, and of erepsin has been described in our earlier paper. At the present time we wish simply to record two negative experiments that may have some interest for other workers. Reimann and Fritsch (10) have reported as much as a 30-fold increase in the potency of whole liver by its digestion with normal gastric juice. This effect was also observed by Fouts, Helmer, and Zervas (11) but to a much less marked degree, and Flood and West (12) have observed a similar effect with liver subjected to the action of normal gastric juice in which pepsin has been destroyed by the action of alkali. The mechanism of this effect is not clear. Since pure crystalline pepsin has very little effect on the active liver product, it seemed to be worth while to determine whether depepsinized gastric juice had any effect on our purified liver product. Accordingly fresh normal gastric juice (25 cc.) ob-

tained by histamine stimulation was brought to pH 10 for half an hour and then brought back to neutrality. It was then incubated with 100 mg. of our purified product and at intervals up to 48 hours aliquot parts were examined for evidence of increased amino nitrogen or carboxyl groups. Since no increase in either carboxyl groups or amino nitrogen occurred, it appears certain that depepsinized gastric juice does not hydrolyze our substance.

It is generally believed that the curious phenomenon known as "plastein" formation is concerned with the interaction of rennin and albumoses. Since our product possesses many of the properties of an albumose, it appeared worth while to see whether rennin had any effect upon it. Using commercial rennin of high activity in neutral and faintly acid solution, we find that it exerts no action on the liver product.

Although not directly concerned with the immediate object of this paper, we may record the fact that we have investigated the possibility that pepsinogen as distinct from pepsin might be concerned in the action of Castle's intrinsic factor which is generally regarded as a necessary precursor of the active liver substance. Through the kindness of Doctors Northrop and Herriott, we have been able to secure highly purified pepsinogen preparations which on clinical testing have proved to be inert so far as a reticulocyte response is concerned, whether administered orally with marmite, or subcutaneously. Reference may be made to old observations by Glaesner (13) on a ferment which as regards distribution and conditions of action closely simulates Castle's intrinsic factor. This enzyme which Glaesner describes as "pseudopepsin" clearly demands renewed investigation.

Brief reference must be made to the results of other workers. Subbarow and Jacobson (14) still adhere to the view that at least three chemically distinct fractions are necessary for an optimum clinical response. They identify these as tyrosine, a complex purine, and a biuret-positive peptide. Since tyrosine and purines are not in any of our preparations, it is hard to correlate their results with ours. On the other hand Strandell (15) has described the clinical results of a preparation which apparently far surpasses ours in activity, for maximal responses are claimed for 2 mg., though it should be noted that some of Strandell's material when tested by Wilkinson (3) showed rather less activity than our

present product. Wilkinson has also described more active preparations, but since no details of preparation or properties are given, we feel that it is impossible to discuss the results with profit.

Since this paper was submitted for publication we have seen the chemical report by Laland and Klem (16) on the preparation of the highly active product which has been tested clinically by Strandell, Poulsson, and Schartum-Hansen (17). The method which is described in principle rather than in detail involves adsorption on active "coal" (charcoal?) followed by elution by phenol, and the subsequent use of organic solvents and also salting-out with ammonium sulfate. Laland and Klem's final product presents certain definite similarities and differences from ours and the recognition of these will certainly be of value in future work. For example, our own product like Laland and Klem's is readily adsorbed by active charcoal. Thus we find that a 1 per cent solution (100 cc.) stirred for half an hour with 1 gm. of active charcoal is adsorbed to the extent of 42 to 45 per cent, with 2 gm. of charcoal 76 to 83 per cent is adsorbed, while with 3 gm. of charcoal adsorption is virtually complete. On elution of the charcoal with liquefied phenol at least 80 per cent of our product may be recovered. On the other hand, elution of the charcoal with 60 per cent alcohol, with 0.2 N hydrochloric acid or disodium hydrogen phosphate does not liberate significant amounts of our product. Commercial albumose, *e.g.* Witte's peptone, shows the same behavior towards charcoal and phenol, so that the procedure is in no sense specific for the liver product. We have been able to make a clinical test of our material that had been subjected to adsorption on charcoal and elution by phenol. 25 mg. given to a patient with an initial red blood cell level of 1.7 millions gave the satisfactory increase of reticulocytes to 19.7 per cent on the 5th day. The fact that Laland and Klem's product is salted-out at least in part by ammonium sulfate is also in agreement with our results, as is also the formation of acid, basic, and neutral amino acids on hydrolysis, although none of them is identified by Laland and Klem. On the other hand, Laland and Klem describe their product as having definitely acid properties, as being bright yellow-red in color, and giving a positive Tollens' reaction for pentose, and containing significant amounts of sulfur. Our product, on the other hand, is essentially neutral, is virtually colorless, gives

no trace of a pentose reaction with Tollens' test, and contains no sulfur.

In an earlier paper in this *Journal* (1) it was stated that "apart from the solubility properties of our preparations . . . there is almost no point of agreement between the properties of the purified preparations described by Cohn, McMeekin, and Minot . . . and our own. For example . . ." As the points of differences were but briefly mentioned and as there was no reference to Cohn's last paper on this subject (18), which had escaped our notice, it may be useful for future workers to note the following quotations. From Cohn's earlier papers: "... the active principle effective in pernicious anemia is not an amino acid but a nitrogenous base, the nitrogen of which exists as in a secondary or tertiary amine" (19); "The active solution gave no precipitate with trichloroacetic acid . . . no increase in amino nitrogen on hydrolysis . . ." (20); "Combined with acetic acid, it dissolved in such organic solvents as chloroform and carbon tetrachloride" (20); "... an active concentrate from which proteoses, peptones, and polypeptides had been removed, and suggested that the active principle was a base rather than a peptide" (19). From Cohn's last paper (18): "... the active principle is a relatively small, predominantly basic . . . compound"; "The basic nature of the molecule was demonstrated by its precipitation by phosphotungstic acid, by mercuric acetate and by tannic acid in neutral solution"; "Beside being free from proteins, carbohydrates and lipoids, it appeared to be free from tryptophane and tyrosine, *arginine*² . . ."; "The very small amount of . . . amino-nitrogen indicated by the Van Slyke nitrous-acid method . . . suggest[s] that we are not dealing with an alpha amino-acid but an ω amino . . . acid"; "... picric acid failed to precipitate the active principle until after the removal of the alpha amino-acids." These quotations make obvious the dissimilarity between Cohn's chemical findings and our own.

As before, the responsibility for the larger part of the chemical work rests with one of the authors (H. D. D.), while the other authors were solely responsible for the clinical tests. Our thanks are again due to all of those to whom we made acknowledgment

² The italic is ours.

in our first paper. To Messrs. Eli Lilly and Company we are indebted for generous supplies of their liver extract, and for preparing kidney and brain extracts for us. Our thanks are also due to Armour and Company who through the kindness of Dr. Fenger supplied us with salivary gland preparations.

SUMMARY

Confirming our previous work, we find that the hematopoietic substance in liver is, or is associated with, a peptide, possessing many but by no means all the properties of an albumose. Further purification of our earlier preparations has led to the conclusion that an aminohexose (glucosamine) is not an essential component of the active molecule, for we have obtained by methods described in detail clinically active products containing no trace of glucosamine. The clinical potency of our glucosamine-free peptide is about twice that of our earlier products. On hydrolysis the peptide yields arginine, leucine, glycine, proline, hydroxyproline, aspartic acid, and an acid resembling hydroxyglutamic acid. Ultrafiltration experiments with graded membranes indicate a molecular size of about $2.1\ m\mu$, and a molecular weight greater than 2000 and less than 5000. Experiments on the effect of alkali (ammonia and sodium hydroxide) are described, and results of the systematic salting-out of the substance with ammonium sulfate.

Depepsinized gastric juice does not hydrolyze our peptide, nor is it concerned with plastein formation by the action of rennin.

Kidney and brain do not yield a hematopoietic substance when subjected to the methods which were successful in the case of liver. The feeding of salivary gland tissue also failed to show clinical activity.

Clinical Tests

The following clinical tests are arranged in the order in which they are referred to in the text. In order to economize space, clinical histories are not included, nor are our negative results with the peptides from kidney, brain, with salivary glands, and with pepsinogen. The dose of peptide material from kidney and brain was 150 mg., given intramuscularly, derived from 600 gm. of kidney and 1250 gm. of fresh brain respectively. The salivary

glands were desiccated at low temperature and defatted with acetone. 10 gm. per day of the ground product were fed for a period of 10 days to a suitable case without effect. In all other cases the material was given by intramuscular injection in a single dose, except in Cases 7, 14, and 15, to whom daily injections were given.

Our thanks are due to Dr. Paul Reznikoff of the New York Hospital, to Dr. Thomas Fitz-Hugh, Jr., of the University Hospital, Philadelphia, and to Professor E. J. Wayne of the University of Sheffield, England, for permitting us to treat patients on their services.

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Day	Case 1		Case 2		Case 3		Case 4	
	Red blood cells millions *	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent	Red blood cells millions	Reticulocytes per cent
1		0.8	1.2	2.3	1.5	2.5	1.1	1.3
2		0.8	*	1.8	*		*	
3				1.3				1.9
4	1.6	1.6		2.2				1.2
5		2.3		2.5				2.1
6		2.8		8.6				
7		5.8		6.5	16	16.7		17.0
8		10.1		12.2	1.9	23.5	1.2	15.6
9		7.8		7.6		22.2		13.7
10			1.5†	3.6			†	6.8
11	1.7†	4.8		2.7				7.3
12		2.5		2.8				5.7
13		2.7		2.4				
14		2.6					1.5	14.2
15	1.7	2.4		5.2				18.9
16	†	2.0		8.3				20.8
17		3.8		9.8				12.5
18		6.2	1.7	6.5				7.8
19		6.0	†	4.8			†	4.8
20		15.2		2.9				
21		23.1		4.1				3.9
22		31.2		15.2				1.9
23		22.7		24.2				4.6
24				28.3				4.1
25	2.5	15.4	2.4	28.3				7.2
26				28.1				9.3
27				22.5				8.0
28								11.5
29			2.6	6.9			†	5.3
30								5.2
32	2.8	3.9						

<p>Subsequent reticulocyte rise to 7% on 40 mg. Preparation 27, uranium filtrate. Response moderate</p> <p>* 100 mg. Preparation 7-A, residue dialysis. Moderate response</p> <p>† 100 mg. No. 3, lead ppt. Probably negative</p> <p>‡ 100 mg. No. 13, lead filtrate. Excellent response</p>	<p>* 25 mg. Preparation 31, uranium ppt. Response slight</p> <p>† 58 mg. No. 43, lead and NH_3 ppt. Response slight</p> <p>‡ 33 mg. No. 44, uranium ppt. Response excellent</p>	<p>* 40 mg. Preparation 19, uranium ppt. Response excellent</p>	<p>* 15 mg. Preparation 25, uranium ppt. Response moderate</p> <p>† 30 mg. No. 25. Response excellent</p> <p>‡ 60 mg. No. 25</p>
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Day	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions
1	2.2	1.5	4.8	1.4	1.0	0.6
2	1.6	1.3	3.4	1.0	1.2	1.1
3	4.4	1.7	4.4	4.3	5.0	2.2
4	7.8	6.4	5.0	6.7	15.0	6.3
5	8.6	8.6	10.2	12.1	7.0	14.8
6	11.5	11.3	14.2	14.2	10.0	17.1
7	18.3	11.3	14.2	18.4	15.5	17.1
8	21.4	13.8	22.0	18.4	1.5	14.5
9	16.2	13.8	21.6	4.5	6.9	11.2
10	6.8	7.0	15.0	1.6	4.8	9.1
11	13.7	2.2	12.2	2.8	11.0	6.4
12				7.4	19.9	2.2
13				17.7	28.9	
14				20.4	22.0	
15				18.9	14.8	
16				11.0	9.8	
17				5.3	8.0	
18				1.9	2.3	
19						
20						
	* 25 mg. Preparation 31, uranium ppt. Response moderate	* 25 mg. Preparation 31, uranium ppt. Response moderate	* 4 mg. Preparation 31 daily, uranium ppt. Response excellent	* Preparation C 50 mg. Response slightly 200 mg. Response excellent Without further therapy, r.b.c. 3.2 millions 30 days later	* Preparation B 25 mg. Response slightly 5 mg. daily. Response excellent	* Preparation B 50 mg. Response moderate

Day	Case 11	Case 12	Case 13	Case 14	Case 15	Case 16
	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions	Red blood cells millions
1	6.1	2.3	1.1	0.4	1.2	0.8
2	5.1	1.8	1.7	0.8	1.0	4.0
3	4.7	1.7	1.2	1.2	0.8	3.0
4		2.8	1.8	1.8	1.2	4.8
5	3.1	5.5		2.0	4.0	
6	4.1	5.5		3.4	3.2	
7	18.2	9.4	19.5	5.4	6.8	
8	12.7	12.6	24.5	6.6	7.8	
9	10.5	6.6	21.5	4.4	7.8	
10	16.4	4.6	9.3	2.4	2.4	
11	10.1	6.6	3.8	9.3	6.7	
12	7.0	6.6	5.1	5.1	5.1	
13	5.9	20.0	5.1	4.5	3.7	
14	6.2	31.8	2.4	2.4	6.5	
15	8.8	31.6	2.9	3.1	6.2	
16	7.6	25.0	3.1	3.1	4.0	
17			2.7	2.7	2.2	
18			1.9	1.9	1.5	
19						
20						
21						
	* 25 mg. Preparation 45, 50% ammonium sulfate † 50% Na ₂ SO ₄ 50% ammonium sulfate	* 25 mg. Preparation 45, 50% ammonium sulfate † 50% Na ₂ SO ₄ 50% ammonium sulfate	* 100 mg. Preparation 45, 50% ammonium sulfate 14 days later, r.b.c. 2.8 millions	* 5 mg. daily Preparation 46, 50% ammonium sulfate † 5 mg. daily Na ₂ SO ₄ 50% ammonium sulfate. Response slight	* 5 mg. daily Preparation 46, 50% ammonium sulfate † 5 mg. daily Na ₂ SO ₄ 50% ammonium sulfate. Response slight	* 12 mg. Preparation 14(D), 50% ammonium sulfate. Response slight Commercial extract with rise to 8% reticulocytes and 2.5 millions r.b.c. in 10 days

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